COMPARATIVE EFFICACY OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS

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Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2008

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DECLARATION

I hereby declare that the thesis entitled **"COMPARATIVE EFFICACY OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"COMPARATIVE EFFICACY OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS"** is a record of research work done independently by **S.Sulficar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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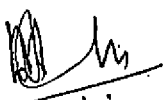
We, the undersigned members of the Advisory Committee of **S.Sulficar,** a candidate for the degree of Master of Veterinary Science in Preventive Medicine, agree that the thesis entitled **"COMPARATIVE EFFICACY OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS"** may be submitted by **S.Sulficar,** in partial fulfilment of the requirement for the degree.



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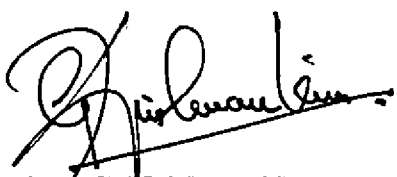

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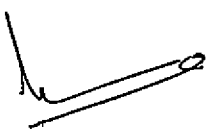

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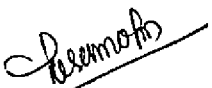

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ACKNOWLEDGEMENT

I have accumulated many debts of gratitude during my work on this manuscript. First and foremost, I would like to express my profound gratitude to ***Dr.M.R.Saseendranath,*** *Professor and Head, Department of Veterinary Epidemiology & Preventive Medicine, and Chairman of the advisory committee, for his exceptional guidance, sustained encouragement, constant supervision and help rendered in all possible ways throughout the course of my study. It gives me immense pride to be the ward of a best teacher award winner; He has been a treasure trove of wisdom for me. His deep insights, determination and dedication helped me a great deal in widening the scope of this work. I benefitted greatly from his frequent interactions and constructive feed back. Without his unstinted support and cooperation successful completion of this work would not have been possible. I owe him a lot.*

I am deeply indebted to ***Dr.G.Krishnan Nair,*** *Professor and Head, Department of Veterinary Microbiology, and member of the advisory committee for his invaluable suggestions and constructive guidance, which helped a lot in the completion of this work.*

I record my sincere gratitude to ***Dr.P.V.Tresamol,*** *Assistant Professor, Department of Veterinary Epidemiology & Preventive Medicine, and member of the advisory committee for extending incessant help and guidance from initiation of the work to completion of the manuscript.*

My inexplicable gratitude goes to ***Dr.Usha Narayana Pillai,*** *Associate Professor, Department of Clinical Veterinary Medicine, and member of the advisory committee for her personal attention, keen interest and affectionate encouragement throughout the tenure of the study.*

I place on record my gratitude to ***Dr.K.Vijayakumar****, Associate Professor, Department of Veterinary Epidemiology & Preventive Medicine, for his insightful suggestions.*

I am grateful to ***Dr.E.Nanu****, Dean, College of Veterinary & Animal Sciences, Mannuthy, for the facilities provided for this research work.*

I am deeply indebted to ***Dr.K.C.Raghavan****, Professor, Department of Animal Breeding, Genetics & Bio statistics, for permitting me to utilize the facilities of University Sheep and Goat Farm, Mannuthy, and specially for arranging Malabari goats from field for completion of my research work.*

My sincere thanks are due to ***Smt.Sujatha*** *Professor, Department of Statistics, for extending help and guidance in the analytical aspects of the data.*

I place on record my deep sense of gratitude to ***Dr.P.Ramadass****, Professor and Head,* ***Dr.Mahalinga Nainar****, Professor,* ***Dr.T.M.A.Senthil Kumar****, Associate Professor,* ***Dr.Kumanan****, Professor and* ***K.Shrine Nagalakshmy****, Senior Research Fellow, Department of Animal Biotechnology, Madras Veterinary College, for their continued support, help and invaluable technical advice with molecular biological technique. This work could not have been completed with out their help.*

My sincere thanks to Government of Kerala, ***Dr Subbayyan IAS****, Secretery to Government for awarding deputation for post graduate studies. I record my sincere gratitude to* ***Dr.R.Vijayakumar****, Director of Animal Husbandry,* ***Dr.K.G.Suma****,* ***Dr.K.Udayavarman****, Additional Directors,* ***Dr.N.N.Sasi****, Registrar,* ***Dr.Shamsudeen Rawther*** *and* ***Dr.Charles****, District Animal Husbandry Officers, Pathanamthitta, other Officers and Staff of Government Secretariat, Directorate of Animal Husbandry and District Animal Husbandry office Pathanamthitta. They all have been very kind to create an opportunity to undertake my PG studies.*

Special thanks are due to all my Teachers and Professors for their effort to educate and stimulate me.

*I am grateful to **Dr.T.V.Aravindakshan, Dr.Joseph Mathew, Dr.Narayanan, Dr.JohnMartin ,Dr.Syam.K.Venugopal, Dr.AjithKumar** and **Dr.Muhamed** for their encouragement, friendship, help, suggestions and support.*

*My sincere thanks to **Sivarajan** for many long days in the field, with goats in all sorts of weather.*

A bouquet of thanks to the helpful library staff, for locating old journals.

*I gratefully acknowledge the help and cooperation of **Smt.Thankam** during my study.*

*It is with affection and appreciation that I acknowledge my indebtedness to **Dr.Nimisha, Dr.Rishi Kesavan** and **Dr. Janus**, my seniors, for always being there with me in times of doubt.*

*Special thanks are due to **Dr.Archana.S.Nair,** my colleague and I acknowledge with gratitude the many days she devoted to work in farm. Above all it was a team effort in which willing help and friendship were always available.*

*Special thanks are also to **Dr.Midhun, Dr.Rojan, Dr.HariNarayanan, Dr.Ganesh, Dr.PrinceMon, Dr.Thomas.K.Thomas, Dr.Aslam, Dr.Nishanth, Dr.Rajeev Dr.Gireesh, Dr.Rajeswari, Dr.Seena, Dr.Indu, Dr.Elso, Dr.Sunil, Dr.Sathyaraj, Dr.Hamsa, Dr.Kanaran,Dr.Unnikrishnan, Dr.Selvakumar, Dr.Binoy** and **Dr.Ayoob** for their support, friendship, encouragement, constant optimism and enthusiasm. Their friendship was like light house whenever I lose my way.*

I am also indebted to UG students of 2002 and 2003 batch, too many to mention all by name, who were wonderful in handling the goats and assisting me in the whole work.

*I am thankful to **Dr.Nimmy** and **Dr.Jincy** for their helping hands in farm.*

*I owe a large debt of thanks to **Dr.Justin Davis** and **Dr.Selvam** for their help, in collecting literature on Johne's disease from IVRI Izatnagar and Madras Veterinary College. Chennai.*

1. INTRODUCTION

Paratuberculosis is a chronic infectious disease of ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). A German veterinarian Dr.H.A.Johne, first described it in a dairy cow in 1895 and the disease was named after him. Because of its long incubation period, high economic losses, difficulties in diagnosis and possible links to Morbus Crohn in humans, paratuberculosis is one of the most important diseases of ruminants today.

Johne's disease primarily occurs in domestic and wild ruminant species most commonly in cattle, sheep, goat, bison, deer and llama. It has also infrequently been reported in rabbits, primates, fox and stoat. The disease is being recognized with increased frequency in goats (Stehman,1996) and when established in goat flocks can cause heavy losses to the farmers.

The first incidence of Johnes disease was recorded in Lahore of the then undivided India by Twort and Ingram (1913). Since then a large number of cases have been reported from different parts of the country and world. Paratuberculosis among goats was reported by Pande (1940b) for the first time in India from Assam .The infection due to MAP has been recorded in almost all sheep and goat rearing states of the country.

The long incubation period, lack of efficient diagnostic tests and the biology of the disease makes diagnosis challenging, especially those in early stages of infection. The disease is difficult to be diagnosed in pre clinical stage, particularly in young animals and such animals act as potential spreaders of the infection during this stage. The animal may not appear ill or produce consistent, specific and long lasting immunologic signs of the infection until months after the infection occurs. The results may be negative although the animal is truly infected. For instance, a false negative faecal culture test result may occur since the organism is shed only intermittently.

On a more personal note, I owe an eternal bill of gratitude to my cousins ***Kabeer, Nazeer, Shefiq, Nazar and Shaji*** *and my brothers in law* ***Ayoob and Nahas*** *who have been the source of inspiration behind my PG study.*

Thanks to my parents for their unending support, encouragement and love.

And most important, my heart felt gratitude goes to ***Sheeja*** *for her empathetic nature, spirits and love. She was instrumental at every stage along the way keeping the whole process on track in the midst of busy schedule. Last, but by no means least, I would like to thank my son* ***Abid*** *and daughter* ***Diya*** *for their patience, love and unconditional support throughout the writing of this thesis.*

I may have left out names of some people who helped me along the way. If so, I am sorry. Please know that I really appreciate all of you. I am truly grateful for the many hands and many hearts that made this manuscript possible.

Above all, I bow my head before the ***Almighty*** *for the innumerable blessings showered on me in all the paths of my life.*

S.Sulficar

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Introduction

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Another example is a negative blood test result for an animal with Johne's disease. This false negative result usually occurs because the antibody is not produced by the goat until late in the disease. Clinically the only sign of *Mycobacterium paratuberculosis* infection may be weight loss in the face of a good appetite. Diarrhoea is not a frequent sign of Johne's disease in goats, although it can occur in some cases.

The insidious nature of paratuberculosis, the lack of a practical and accurate diagnostic test and the failure of many farmers to recognize the disease among their stock have made epidemiological surveys difficult. Presence of large number of healthy carriers among the infected herds complicate control of paratuberculosis. Early detection and removal of such subclinical carriers are now recognized as the most important aspect of control of paratuberculosis. Diagnosis of the infection in goats is more difficult than in cattle.

Intra dermal test was first developed by Vonpriquet in 1907 (Skinner, 1949) for diagnosis of tuberculosis and the technique was later adopted by Bang (1909) for the diagnosis of paratuberculosis. Intradermal Johnin test has been widely used under field conditions. Johnin test is helpful in identifying especially the early stages of the disease but in later clinical stage, animals may not evoke enough response. This assay may simply indicate exposure and presence of stimulated memory cells (Heitala, 1992) and is not a very reliable test but can be considered as a test of indication. Reliability and specificity of Johnin test has been a matter of debate since the inception of its development (McFadyean *et al.*, 1916).

Microscopic examination of faecal samples of suspected animals by acid-fast staining may reveal mycobacterial bacilli, but the sensitivity of this method is low. An additional draw back is that accurately distinguishing *Mycobacterium paratuberculosis* from the non pathogenic mycobacteria (saprophytes) commonly occurring in such samples can be difficult even for experienced technicians , resulting in low specificity of this diagnostic method.

Due to the delayed confirmatory diagnosis, often the control programme becomes difficult .The early detection will help to cull all the positive animals from the herd, thus reducing the spread of infection as a part of the control measure.

The present study was conducted with following objectives.

1. For the assessment of comparative efficacy of various tests for the early diagnosis of paratuberculosis in goats.

2. For recommending a more suitable diagnostic test for paratuberculosis in goats.

Review of Literature

2. REVIEW OF LITERATURE

2.1. HISTORY

Johne's disease or paratuberculosis is a chronic, fatal, intestinal mycobacterial infection, characterized by cachexia and in some species diarrhoea, after a long preclinical phase and is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The deficiencies in reliable means of diagnosis, treatment or prevention made control extremely difficult and as a result , the disease continues to spread amongst the world's food animal industries at an alarming rate . The disease is widely prevalent in cattle and has been designated as multi species disease by OIE (*Office International des Epizooties*) (OIE, 2008).

Johne and Frothingham (1895) described a chronic form of bacterial enteritis in cattle . Reports of the occurrence of a similar disease were published in Holland, Denmark, Belgium and Switzerland within a decade. The first case in England was recorded by McFadyean, in 1907, who gave a detailed description of his microscopical findings and introduced the term Johne's disease, by this name it is still known (Glover, 1937). On genetic basis, *Mycobacterium paratuberculosis* is virtually identical to *M. avium*. However, the phenotypic characteristics of the two organisms are different. An opinion supported by the International Association for *Paratuberculosis* was that *M. paratuberculosis* should be reclassified as a subspecies of *M. avium* and thus was renamed *Mycobacterium avium* subspecies *paratuberculosis* (Collins, 2003a).

Paratuberculosis was first described in North America in 1908 (Kreeger, 1991). Twort and Ingram (1913) isolated the organism and named it as *Mycobacterium enteritidis chronicaeparatuberculosae bovis johne.*

McFadyean and Sheather (1916) described the disease for the first time in goats.

Johne's disease came to Iceland in 1933 when 20 sheep of the Karakul breed were imported from Halle, Germany . The first clinical case of paratuberculosis in sheep was

confirmed in 1938 and in cattle in 1944. Within 16 years Johne's disease together with other karakul diseases (maedi and jaagsiekte) almost ruined sheep farming, the main agricultural industry in Iceland (Fridriksdottir *et al.*, 2000).

Doyle and Spear (1951) stated that Johne's disease had been the cause of considerable cattle losses for over 200 years in England.

Johne's disease was first recorded in sheep in 1980, in central New South Wales (Seaman *et al.*, 1981).

In South Africa ovine paratuberculosis was unknown until an infected Merino ram was imported in 1967. During the 1990 decade paratuberculosis among sheep farms in Western Cape became wide spread (Michel and Bastianello, 2000).

2.2. PREVALENCE OF PARATUBERCULOSIS

2.2.1. The global scenario

Paratuberculosis was first described in 1895 by Johne and Frothingham in Europe, and its presence was subsequently reported world wide.

Hole (1952) based on surveys of abattoir, in England during 1949 reported a prevalence rate of 11 per cent for paratuberculosis in cattle.

In the later 1950's the prevalence of this disease in cattle in UK was 0.6 per cent (Withers, 1959).

Sherman and Gezon (1980) reported that the annual incidence due to *Mycobacterium paratuberculosis* in goat herds have been as high as 11.1 per cent.

One national prevalence study revealed that 1.6 per cent of dairy and beef cattle were infected with *Mycobacterium avium* subsp *paratuberculosis* in United States (Merkal *et al.*, 1987).

Collins *et al.* (1994) in a survey of dairy herds in Wisconsin yielded an estimate of 34 per cent infection rate in the herd, based on serological diagnosis.

In the Madrid region in Spain a serological prevalence study was conducted predominantly in sheep and some goat flocks and the true prevalence rate was estimated to be 44 per cent (Mainar-Jaime and Vazquez-Boland, 1998).

Result of a survey indicated that the disease was wide spread in Netherlands . Herd prevalence of Johne's disease in Europe was reported to be between 7 and 55 per cent , in the US nearly 40 per cent and in Australia it ranged between 9 and 22 per cent (Manning and Collins, 2001).

Herthnek (2006) stated that a few countries including Sweden had very low prevalence. For three centuries there were no reports of paratuberculosis in Sweden , until four cases were found in beef cattle in 1993. Investigations identified a total of 53 infected cattle herds, all linked to animal imports. All animals in the herds were culled, in accordance with Sweden's stamping-out policy.

In Belgium seroprevalence was estimated to be six per cent by ELISA. In Denmark a serological study of bulk-tanked milk from 900 dairy herds reported that 70 per cent of herds tested positive for MAP infection. In Argentina an epidemiological investigation demonstrated a reactivity of 26.5 per cent for beef cattle and 56 per cent for dairy cattle. In Brazil ELISA showed 18 per cent positive results in 82 per cent of the farms (Lilenbaum *et al.*, 2007).

2.2.2 Incidence in India

2.2.2.1. In Cattle

Systematic studies on the prevalence of Johne's disease in India have been scarce. However the disease is widely prevalent amongst cattle, buffaloe, sheep and goats (Tripathi *et al.*, 2002).

In India, the first case of Johne's disease in cattle was observed in Lahore of undivided India (Twort and Ingram, 1913), followed by another case in 1917 from a

military dairy farm. Since then large number of cases had been reported from every part of the country and many cattle farms had suffered heavy losses due to this disease.

A comprehensive study of bovine paratuberculosis was carried out by Pande (1940a) during 1933-1937 in Assam. From Assam first case of Johne's disease was diagnosed in a Sindhi bull at Khanapara government cattle farm in 1933. During this investigation, he reported the incidence of the disease to be varying between 10 and 66 per cent, based on the avian tuberculin test. During this period disease was also diagnosed in cattle of Mukteswar dairy farm by avian tuberculin test, along with the demonstration of acid fast bacilli in faeces, tissue smears and bowel washings.

Rajagopalan (1947) reported 14 per cent disease prevalence at Mukteswar goverment cattle farms, based on Johnin testing.

Rao (1950) made a systematic investigation of Johne's disease in two cities *ie*, Bangalore and Mysore. In Bangalore 4 per cent and in Mysore 1.5 per cent cattle were found to be suspected for Johne's disease.

The incidence of paratuberculosis in cattle of Rajasthan was found to be 1.78 per cent (Purohit and Mehrotra, 1969).

During 1970 to 1979, 3.6 per cent of 73 buffaloes were found positive for Johne's disease in Haryana . Study from Haryana revealed that 1.9 per cent of 4758 cattle reacted positively to paratuberculosis infection (Kulshrestha *et al.*, 1980).

According to Bapat and Bangi (1985), 1.8 per cent of 1826 cattle were positive on Johnin skin test examination in Maharastra.

Paliwal *et al.* (1985b) reported Johne's disease in 32 cattle, belonging to Jersey, Holstein, Brown Swiss and their crosses, by different methods such as Johnin testing, smear examination of faeces and rectal pinch, indirect immunofluorescence test and histopathology.

Mukhopadhyay *et al.* (2001) when tested forty one animals in a Pondicherry cattle farm against Johne's disease by inoculating Johnin in the neck, found fourteen animals positive.

In a study involving 26 samples from dairy cattle, Johne's disease was diagnosed in 84.6, 96.1, 88.4 and 23 per cent animals by faecal culture, milk culture, milk ELISA and milk PCR respectively (Singh *et al.*, 2007a).

Sivakumar *et al.* (2007) based on an absorbed ELISA found that serological prevalence of Johne's disease in buffaloe was 14.5 per cent.

2.2.2.2. In Sheep and Goats

Rajagopalan recorded the disease among goats at Mukteswar during 1947-1948.

In an organised farm in Uttarpradesh, 18 per cent of 423 sheep showed para-tuberculosis infection on the basis of faecal examination (Paliwal *et al.*, 1984).

Sharma *et al.* (1985) noted that in Punjab, 3.5 to 16 per cent of 835 sheep belonging to different breeds at a farm were found reacting to Johnin.

The over all prevalence of Johne's disease was 8.7 per cent when 1976 goats were tested with intradermal Johnin between 1978 and 1985 in Maharashtra (Srivastava and More, 1987).

Kumar *et al.* (1988) reported that on post-mortem examination of 3334 goats between 1972 and 1986 the overall incidence of paratuberculosis was 8.76 per cent and most of the animals were 1.5 to 5 years of age.

In a study of 998 sheep of various breeds from different parts of Rajasthan state , the highest incidence of *Mycobacterium paratuberculosis* was in Sikkar (33.8 per cent by rectal pinch smear staining and 42.3 per cent by intradermal testing) (Jatkar *et al.*, 1990).

Tripathi and Parihar (1999) on necropsy of 1117 goat carcasses, found the overall incidence of paratuberculosis as 3.5 per cent and the highest incidence of 2.2 per cent was reported in the age group of 1-2.5 years. Occurrence of paratuberculosis in three kids below one year of age indicated the possibility of infection of kids early in the life or from the infected dam through intrauterine transmission.

2.2.3. Kerala scenario

Regarding the incidence and prevalence of Johne's disease, no systematic study has been undertaken in Kerala so far. The disease has not had the intensive investigation its importance demands. Study conducted by Vinodkumar *et al.* (1999) revealed that the disease was prevalent in goats.

2.3. ETIOLOGY

Mycobacterium paratuberculosis is difficult to cultivate in primary cultures, it had originally been grown only in media containing heat killed acid fast bacteria (Twort and Ingram, 1913). *Mycobacterium avium* subsp *paratuberculosis* is traditionally distinguished from the ubiquitous *M. avium* by its extremely slow growth in culture and requirement of exogenous mycobactin for *in vitro* growth. Mycobactin is a high molecular weight complex lipid that chelates iron for storage in bacterial cell wall (Sneath *et al.*, 1986).

Mycobacterium avium subsp *paratuberculosis,* the causative agent of Johne's disease is a hardy, slowly growing, facultatively anaerobic, acid fast, partially gram positive bacterium, a member of *Mycobacterium avium* complex. They are non motile , non sporing , non capsulating and appear as clumps in smears. The organism is an intracellular pathogen surrounded by complex tripartite lipid rich cell wall. Cell wall contains N-glycolyl muramic acid and this renders their surface hydrophobic and makes mycobacteria difficult to stain with dyes at room temperature. Bacteria takes stain with dyes on prolonged application or heating. When the bacteria are stained they resist decolourization with 1 per cent Hcl and 95 per cent ethanol. For this reason they are referred as acid fast organisms. They are short rods measuring 0.5µm width and 1 to 2µm in length (Quinn *et al.*, 2002).

2.4. TRANSMISSION

2.4.1. Faecal excretion and oral infection

Sometimes a passive excretion occurs after oral infection. In natural situation positive faecal culture in the absence of other evidence of infection at necropsy suggests passive excretion. Such findings have been reported in goats by Whittington *et al.* (1998).

In some situations passive excretion have significant epidemiological consequences. Previously uninfected animals briefly exposed to high levels of environmental contamination may excrete MAP and present a risk to other stock for up to a week. There after such animals are likely to have been infected at the same time and may thus present a further risk when later they begin to excrete the organisms themselves (Whitlock *et al.*, 2000).

Whittington *et al.* (2000) found that sheep infected with the multibacillary form of the disease were shown to excrete 10^8 organisms per gram of faeces, measured by end point titration in Bactec culture. In cattle viable unit counts on solid media of about 10^6 per gram have been measured. Thus environmental contamination from even a single clinical case may be considerable. Moreover MAP has been shown to survive for considerable period in the environment so that levels of environmental contamination might build up over time. While clinically affected animals are likely to represent the greatest risk to herd mates, subclinically infected animals also shed MAP in faeces, and infection may persist in a herd or flock with few or no observed clinical cases. Excretion can occur from animals that never become clinical cases or later eliminate the infection entirely. Faecal shedding of MAP was observed sporadically for the first six months after experimental infection in calves, which showed no clinical signs up to twenty months.

Whittington and Sergeant (2001) reported that exposure to organisms originating in faeces can occur by ingestion of contaminated pasture, soil or water or from faeces on the teats. Clinically affected cattle, sheep or goat can excrete enormous number of organisms

in their faeces. Faecal excretion is the main source of environmental contamination with MAP and is probably the main means of transfer of infection between animals

2.4.2. Infection through milk

Streeter *et al.* (1995) in a study observed that of the 36 faecal culture positive cows, *Mycobacterium paratuberculosis* was isolated from colostrum of eight cows and from milk of three cows. Cows that were heavy faecal shedders were more likely to shed the organism in the colostrum than light faecal shedders.

Clinically affected cows were more likely to excrete MAP in milk with a prevalence of 45 per cent (Giese and Ahrens, 2000).

Grant *et al.* (2000) using PCR detected MAP DNA in 88 per cent of milk samples from sheep with positive IFN-γ tests.

Raizman (2007) demonstrated that cows with clinical paratuberculosis did shed viable organisms in their milk at low levels (50 CFU / 50ml of milk). Colostrum was available to the neonate at the time of highest susceptibility to infection.

2.4.3. Congenital infection

Doyle (1958) observed foetal or uterine infection from clinically affected cows and rate of foetal infection from such animals was as high as 26 to 35 per cent. Congenital infection of the foetus has been demonstrated frequently in cattle.

Foetal tissue from five of fifty eight cows were culture positive for *Mycobacterium paratuberculosis.* All five culture positive foetuses were from cows that were classified as heavy faecal shedders, indicating that foetal infection was found only in cows that were heavy faecal shedders (Sweeney *et al.*, 1992).

Lambeth *et al.* (2004) observed that intrauterine and trans mammary transmission of MAP might occur frequently in clinically affected sheep and these were less common in

subclinically infected ewes. All ewes with clinical ovine Johne's disease under study had infected foetuses.

2.4.4. Venereal transmission

Larsen *et al.* (1981) reported that bulls could be genitally infected and shed MAP in semen.

Mycobacterium avium subsp *paratuberculosis* had been detected from the semen of clinically affected bulls by real time PCR (Herthnek, 2006).

Singh *et al.* (2007b) reported that vaginal secretion was a potential source of contamination to newborn kids and animal handlers and considered as a good clinical material to screen post parturient female goats against MAP infection. Thus venereal transmission in either direction was theoretically possible, as is direct infection of a developing embryo, without established infection in the dam. However, such possible infections were probably of minimal significance in the field.

2.4.5. Embryo transfer

Rohde and Shulaw (1990) isolated *Mycobacterium paratuberculosis* from uterine flush fluids from 3 to 4 clinically infected cows and washed ova had been shown to harbour the organism, indicating that embryo transfer from infected donors to uninfected cows was not entirely without risk .

2.5. CLINICAL SIGNS

Moser (1982) reported that diarrhoea in sheep and goat with Johne's disease may be pasty, intermittent, present only terminally or absent altogether.

Manning and Collins (2001) reported following clinical signs, pathological and microscopical lesions. In cattle, weight loss despite adequate rations, accompanied by chronic diarrhoea were standard clinical signs of Johne's disease. Hypoproteinaemia and bottle jaw or dependent mandibular oedema are also reported in cases of advanced disease. However in other species clinical hints of the infection may be limited to the vague and non-specific finding of weight loss. In species with heavy coats (eg; Sheep and Ilamas), this single indicator may be easily missed. Diarrhoea is infrequently observed with paratuberculosis in sheep, goat, bison and perhaps other non-domestic hoof stock species. In the last phase of Johne's disease, animals of any species may become cachectic and too weak to rise.

A range of pathological lesions could be exhibited at necropsy, depending upon the stage of infection and species in question. The classic lesions described in bovine cases included a corrugated and thickened ileum with enlarged and oedematous mesenteric lymph nodes, in addition to distension of lymphoid channels. Classic histopathological findings included an extensive granulomatous infiltrate of intestinal villi, abundant multinucleated giant cells and innumerable intracellular acid fast bacilli. In some case of infection in bison and sheep, only indication of infection might be a single giant cell and sparse or no intracellular bacilli.

Microscopic lesions such as histiocytic or granulomatous inflammation including acid fast bacilli in any tissue of the gastro-intestinal tract and mesenteric lymph nodes were compatible with Johne's disease. Multinucleated giant cell or epithelioid macrophages in the lamina propria at any level of the gastro-intestinal tract should raise suspicions of MAP infection even in the absence of acid fast bacilli.

Reddacliff (2002) observed that classical sign of sheep with severe JD is progressive emaciation.

Clinical signs are not a reliable indicator of the presence or absence of MAP infection in sheep and goats because diseases with similar clinical signs of Johne's disease in small ruminants included chronic intestinal parasitism, internal abscesses such as those caused by

Corynebacterium pseudotuberculosis, chronic hepatic disease and chronic malnutrition. In sheep and goats, diarrhoea is less frequent and occur in younger animal. When diarrhoea does occur, it typically attends end stage disease (National Research Council) (NRC, 2003).

Kruze *et al.* (2006), in a case of paratuberculosis of goat in Chile, noted the classical clinical sign of emaciation despite willingness to eat, dry and rough hair coat and no incidence of diarrhoea.

Emaciation or wasting disease, unresponsiveness to dewormers and antibiotics is the usual sign. Appetite is often good, in spite of weight loss, until the animal is near death. Normal stools are the usual observation even in clinically diseased animals, but intermittent diarrhoea or softened pasty stools are occasionally observed. The profuse watery diarrhoea seen in cattle is not a common feature of the disease in sheep and goats (Shulaw, 2007).

2.6. DIAGNOSIS

2.6.1. Detection of organism

2.6.1.1. Acid fast staining of faecal sample and microscopy

Doyle (1956) stated that only 25-30 per cent of cases were identifiable by first examination of faecal sample. The chief advantage of the method was its accuracy, the presence of typical clump was accepted as indisputable evidence of lesion in bowel wall.

Hole and Maclay (1959) reported that microscopical examination of faecal sample had a marked positive value in clinical disease, (but a negative result had no significance).

Julian (1975) opined that the examination of faeces for acid fast organism had been widely used, particularly to pick out shedders in a known positive herd. The finding of clump and acid fast organism in faeces would be significant.

Moser (1982) demonstrated that examination of faecal smear was less reliable than culture since a greater number of low level shedders might be missed. Since faecal shedding is not a consistant feature of sub clinical infection, faecal culture or smear

examination result in a large number of false negative tests. Repeated faecal testing may detect the intermittent shedder but the process is cumbersome in large herds or flocks.

Chiodini *et al.* (1984) noted that slow progression of the disease and intermittent shedding of MAP in faeces made it difficult to detect infected animals.

Kulshrestha *et al.* (1984) reported that faecal sample examination appeared to be comparatively simpler and better than Johnin test, particularly to detect clinical shedders. However negative faecal test did not necessarily conclude that the animal was free from infection.

Paliwal *et al.* (1984) reported that for the diagnosis of Johne's disease in sheep, faecal sample examination was more reliable than intradermal allergic test.

Kandavel and Nedunchelliyan (1987) reported positivity of 43.4 per cent by faecal examination in 120 cattle of Holstein and Sindhi breeds, for Johne's disease.

Microscopic detection of organism in faeces and mucous membrane of intestine by Ziehl-Neelsen staining is a quick and easy to perform method for the detection of *Mycobacterium paratuberculosis* and reported specificity was 93.8 percent but sensitivity as low between 35 and 46 per cent (Heitala, 1992).

Acid fast staining has limited sensitivity and as many as 10^6 bacteria per gram of tissue may be necessary for detection of acid fast rods in sections by light microscopy. Immunohistochemical technique seemed to be superior to Ziehl-Neelsen staining as a diagnostic method for Johne's disease in goats (Thoresen *et al.*, 1994).

In a study by Egan *et al.* (1999), out of 56 Johne's disease positive cases reported, acid fast bacteria were observed in faecal smear from 13 samples (23.6 per cent).

Zimmer *et al.*, (1999) recorded least sensitivity of 36.4 percent for Ziehl-Neelsen staining after conducting studies in 132 infected cattle and concluded that Ziehl-Neelsen staining had the lowest detection rate and proved unreliable in diagnosing Johne's disease.

Examination of faecal smears stained with Ziehl-Neelsen was an unreliable means of assessing daily excretion in individual animals, except those with severe lesions. Excretion of MAP in Merino sheep with multibacillary Johne's disease occurred daily, proving that

environmental contamination could be continuous on farms with endemic ovine Johne's disease (Whittington *et al.*, 2000).

Calton (2002) observed that in paucibacillary form of the disease *Mycobacterium avium* subsp *paratuberculosis* might not be observed following either acid fast staining or culture. One study showed that in 30 per cent of goats with paucibacillary lesion, no acid fast bacteria were seen in the mucosa of grossly affected sections.

In an outbreak of paratuberculosis complicated with schistosomes in sheep Kataria *et al.* (2004) observed that faecal sample from many of the affected animals did not show presence of organism.

Diagnosis of paratuberculosis can be made if acid fast bacilli are seen in clumps (three or more organism of 0.5-1.5μm size) in Ziehl-Neelsen stained smear of faeces. The presence of single acid fast bacilli in the absence of clumps doesn't indicate a definitive diagnosis. The disadvantage of this test is that only about one third of cases can be confirmed on microscopic examination of a single faecal sample (OIE, 2004).

Direct examination of faeces stained with Ziehl Neelsen method permits the visualization of the bacilli. However simple visualization cannot differentiate MAP from any environmental bacteria. When animals are low shedders, faecal sample examination could also lead to false negative results. For these reasons, direct examination although cheap is not recommended for diagnosis (Lilenbaum *et al.*, 2007).

2.6.1.2 Bacterial culture

The Johne's bacillus was first described in the inflamed intestine of a cow in 1895. The bacillus was eventually grown in 1912 on enriched culture media to which extract of *Mycobacterium tuberculosis* and *M. phlei* had been added (Twort and Ingram, 1913).

Gilmour (1976) reported that the advantage of faecal culture was its specificity and the disadvantage was that it detected only relatively heavy excretors .

Faecal culture has been considered for years as the most sensitive, specific and precocious method for detection of clinical and sub clinical paratuberculosis in cattle. Although it is still a powerful tool for control of paratuberculosis in this species, its extensive use has been hampered by its high cost and by its slow outcome (Chiodini *et al.*, 1984).

Englund *et al.* (1999) found that it was not possible to detect all subclinical infection by culture, because of no or low shedding of *Mycobacterium avium* subsp *paratuberculosis*.

Egan *et al.* (1999) was successful in isolating organism from 39 cows by culture out of 56 Johne's disease positive cows (69.6 per cent).

Bacteriological culture of faeces is the most sensitive herd test. The passage of time and the repeated testing are the greatest allies in detecting paratuberculosis because the infected animals progress in the disease process and most tests are more effective in the later stages of disease (Whittington and Sergeant, 2001).

Collins (2003a) observed that as the infection progressed in the animal, the frequency and number of bacteria being excreted increased.

Culture techniques were refined after the mycobactins, which were iron chelating proteins, were subsequently identified as the essential ingredient from those extracts that enabled cultivation of Johne's bacillus (NRC, 2003).

Sub clinical carriers excrete variable number of *Mycobacterium avium* subsp *paratuberculosis* in faeces. In most cases, large number of organisms were excreted when clinical disease developed (OIE, 2004).

Huntley *et al.* (2005) opined that isolation and culturing of MAP from faeces or tissue remained the most definitive detection method, yet could result in false negative results based on a low level of colonization in tissue or negligible and intermittent shedding of organism in faeces.

Austerman *et al.* (2006) found that faecal culture typically detected animals only in the later stages after they started shedding the organism. For eradication of

paratuberculosis from a flock or a herd, however it was crucial for diagnostic tests to identify the animals before they started shedding the micro organism.

Bacterial culture and serum antibody assays have relatively low sensitivity and hence they have an application in the later stages of Johne's disease (Stewart *et al.*, 2006).

Isolation of *Mycobacterium avium* subsp *paratuberculosis* from tissues and faecal sample is a test with 100 per cent specificity. Tissue culture is more sensitive than faecal culture if samples are collected from sites of suggestive lesions. *Mycobacterium avium* subsp *paratuberculosis* shows the most fastidious growth of all mycobacteria and incubation lasts for 8-12 weeks. The main feature that distinguishes these species from other mycobacteria, is its dependency on exogenous mycobactin J, an iron chelating substance used for *in vitro* cultivation. Cultivation can identify shedder animals, which present more than 100 micro organisms per gram of faeces. The shedding of bacteria is intermittent and it does not occur during the early stage of the disease. Predominantly used culture media is Herrold's egg yolk agar with mycobactin J and needs 8-12 weeks incubation (Lilenbaum *et al.*, 2007).

2.6.1.3. Automated culture- radio metric system

Radiometric culture was more sensitive than histopathology in detecting *Mycobacterium avium* subsp *paratuberculosis* infection in sheep and goats and more sensitive than culture on Herrolds eggyolk medium for the detection of infection in cattle (Whittington *et al.*, 1998).

The radio metric system in greatest current use is the BACTEC system. Bactec is highly automated, faster and apparently has a slightly higher sensitivity than conventional culture system, but it is quite expensive and requires the use of radio isotope (^{14}C-labelled palmitic acid). The instrumentation detects the the ^{14}C labelled CO_2 that is produced by metabolism of the labelled palmitic acid (NRC, 2003).

Bactec culture system uses a liquid broth, Bactec12 with a nutrient, source supplemented by radio isotope CO_2 . Bacterial growth is delected by automated equipment, the Bactec 460 , after 4-7 weeks of incubation. The major problem of this process is its high cost and the disposal of radioactive waste (Lilenbaum *et al.*, 2007).

2.6.1.4. Antigen 85 – monoclonal antibody immuno assay

Research is underway to develop an assay capable of detecting circulating antigen 85, a secreted product of actively replicating mycobacteria. If a monoclonal antibody to *Mycobacterium avium* subsp *paratuberculosis* antigen 85 were to show high specificity and sufficient sensitivity, this ability to detect the infection without relying on an immune response from the host would be most beneficial. The assay would thus offer the potential of detecting animals at the earliest stages of infection, months before any histopathological or clinical evidence of Johne's disease would appear. Efforts are in progress to validate this assay for diagnosis of Johne's disease in both bison and captive wild life (Manning and Collins, 2001).

2.6.2. DETECTION OF HOST RESPONSE

2.6.2.1. Detecting the cell mediated response

Bendixen (1977) reported use of lymphocyte migration inhibition test for the diagnosis of paratuberculosis.

It is now well recognised that immunological resistance and the earliest specific immunological responses to *Mycobacterium avium* subsp *paratuberculosis* infection depend on cell mediated immune mechanisms. In the past, intradermal skin tests and lymphocyte blastogenesis were used more commonly for diagnosis of paratuberculosis (Johnson *et al.*, 1977).

Buergelt *et al.* (1978) reported that tests for cell mediated immunity include the intradermal skin test for delayed type hypersensitivity and lymphocyte stimulation assays.

Goats infected with *Mycobacterium paratuberculosis* revealed more cell mediated immune response in preclinical cases and impaired response in clinically infected animals (Paliwal *et al.,* 1985a).

Intravenous Johnin test has been used in the past, either alone or to prime subsequent lymphocyte stimulation assay (Kormendy *et al.,* 1990).

Rothel *et al.* (1990) described the validity of IFNγ assay. Of these only intradermal test and IFNγ assay have been much used out side of experimental situations.

Inter leukin-2R assay is a recently developed test that showed promise for detection of early infection , but so far this test has been used only in small number of experimentally infected goats (Whist *et al.,* 2000).

2.6.2.1.1. Delayed type hypersensitivity reaction

According to Larson *et al.* (1932) and Lall (1963) the intradermal Johnin test was an efficient and satisfactory test for diagnosis of Johne's disease.

Taylor (1951) observed that testing of infected herds of cattle with Johnin gave 30 percent or more positive results.

Hole and Maclay (1959) reported that reaction to allergic test was most marked in the initial stage of infection and became less marked as the disease was established.

Konst and McIntosh (1958) demonstrated that periodic intradermal testing with Johnin PPD in cattle indicated the value of the test in assisting in the control of Johne's disease from proven infected herds.

Karpinski and Zorawski (1975) found that allergic test with the use of Johnin had a limited diagnostic value and made it possible only to suspect paratuberculosis in the herd.

Skin testing can be quite sensitive soon after infection by *Mycobacterium avium* subsp *paratuberculosis*, which turns declining over time (Gilmour *et al.*, 1978).

Moser (1982) opined that the intradermal Johnin test was widely used to detect cell mediated response to MAP. It was considered unreliable due to a high proportion of false negative results, especially in the later stage of the disease.

In cattle skin testing was now rarely used because it had been found repeatedly to have both low sensitivity and low specificity (Chiodini *et al.,* 1984).

Allergic test appeared to be least reliable in diagnosing Johne's disease in sheep (Kulshrestha *et al.*, 1984).

Intradermal Johnin test, compliment fixation test, microscopic examination as well as examination of faeces by culture were carried out in twenty one cattle from four herds infected with Johne's disease and none of the tests studied in these cases removed all doubts as to whether the animals were or were not subclinically infected with *Mycobacterium paratuberculosis* (Wentink *et al.,* 1984).

For diagnostic purpose Johnin was injected into the caudal fold of 630 sheep and out of these 58 animals were found positive. Acid fast bacilli were identified in faeces from 5 out of 58 sheep (Sharma *et al.,* 1985).

Kandavel and Nedunchelliyan (1987) reported a test positivity of 64.1 percent for Johnin intradermal test, after conducting study in 120 cattle and found that Johnin test was useful in the diagnosis of paratuberculosis.

In a study conducted by Paliwal *et al.* (1987) Johnin test was found to be reliable to the extent of 20.73 per cent in cattle.

Kormendy (1990) in a study noted that out of 230 cows tested , single intradermal Johnin test was positive in 58.1 per cent of faecal positive and in 54.4 per cent of faecal negative cows respectively.

Intradermal skin testing using Johnin antigen is technically uncomplicated but often produces equivocal results with low sensitivity and specificity (Kreeger, 1991).

Goswami and Ram (1998) opined that more extensively used intradermal Johnin test has its own serious limitation of false negative finding, persumably because of anergy.

Vinodkumar *et al.* (1999) demonstrated that modified intradermal test (three days later a second injection of 0.1 millilitre of PPD Johnin was given intradermally exactly on the same site of the first injection and reading was taken 24 hours after the second injection) was better than single intradermal test.

Benedictus *et al.* (2000) reported that in 1931, cattle from Netherlands were tested intradermally with the Johnin in order to trace infected animals at an early stage.

Intradermal allergy test is the only field test available for diagnosis of Johne's disease and TB. Sensitivity and specificity of this test is influenced by stage of disease and immune status of animal. Double intradermal test was more useful compared to single intradermal test (Sisodia *et al.*, 2000).

Harris and Barletta (2001) stated that testing for MAP was limited to the intradermal skin test. This test evaluated the DTH reaction of an animal to injected MAP extracts and was an indication of the CMI response of the animal.

Amongst the single intradermal and double intradermal tests in buffaloe for detection of Johne's disease, double intradermal test was found to be superior by Sisodia and Shukla (2002).

The skin test takes advantage of the development of delayed type hypersensitivity (DTH) reaction to the intradermal injection of a mycobacterial extract, purified protein derivative (PPD) Johnin (NRC, 2003).

Roy *et al.* (2004) noted that Johnin test was helpful in identifying the early stage of disease, but in later clinical stage animal might not evoke enough response.

In the early stages of paratuberculosis, the cell mediated immune response predominates and then wanes with advancing disease. Two CMI diagnostic tests for paratuberculosis have been proposed for field use. The skin test which measures the delayed type hypersensitivity reaction *in vivo* and interferon -γ (IFN-γ) ELISA which measures IFN- γ production in response to antigen *in vitro*. More than 100 reports have been published on the use of skin testing as a diagnostic tool, largely from 1911-1960's (Austerman *et al.*, 2006).

Study conducted by Tripathi *et al.* (2006) showed that Johnin test was positive in 25 of 34 (73.5 per cent) goats that included 17 multibacillary and 8 paucibacillary cases. Most paucibacillary cases showed stronger DTH reactions.

Study by Antognoli *et al.* (2007) to evaluate the results of intradermal skin test, a modified IFN γ test and commercial ELISA in dairy calves at 2, 4, 6 and 8 months of age relative to faecal shedding of MAP revealed disagreement among all the tests evaluated and suggested that detection of MAP infection in young stock required the use of combined multiple tests.

Considering the various assays for cellular immunity, intradermal allergic test was the very first to be utilised for diagnosis (Lilenbaum *et al.*, 2007).

Shulaw (2007) opined that intradermal Johnin testing might yield many false positive and false negatives. It had been suggested that the absence of any positives in a screening test of all animals might suggest that the herd or flock was not infected.

Singh *et al.* (2007a) stated that success of any disease eradication programme largely depended on the accurate determination of the initial herd prevalence . Single intradermal Johnin test could be used as a preliminary screening test for the diagnosis of Johne's disease.

2.6.2.2. Systemic tests using intravenous Johnin

2.6.2.2.1. Temperature response

Benedictus and Bosma (1985) found that injection of 1.6 ml of Johnin intravenously in clinically suspected cattle aid in diagnosis of paratuberculosis and disease could be confirmed in 96 per cent cases. The sensitivity of intravenous Johnin test was considerably lower in cases of non clinical Johne's disease.

Intravenous Johnin test along with haematological study could be applied in the diagnosis of paratuberculosis in goats (Somvanshi *et al.*, 1987).

Intravenous injection of Johnin produced a temperature increase in sensitized animals. A rise of 1.5° C six hours following injection was significant. This test is said to be the most valuable for differentiating clinical Johne's disease from other causes of chronic diarrhoea. The test requires large volume of Johnin antigen, 2 to 4 ml (Kreeger, 1991).

2.6.2.2.2. Leukocyte response

Julian (1975) reported that intravenous injection of Johnin produced a stress or cortisone type response in sensitized animals, with an increase in neutrophils and a decrease in lymphocytes. A positive result would be indicated when the ratio of neutrophils to lymphocytes six hours after injection was more than twice the ratio of neutrophils to lymphocytes before injection.

Kormendy *et al.* (1990) opined that evaluation of responses elicited (changes in the neutrophil / lymphocyte ratio of blood), by intravenously administered Johnin PPD could be a valuable aid in recognizing infected cattle particularly those among the heifer progeny of infected cows.

2.6.2.2.3. Gamma-Interferon assay (IFN-γ)

Cell mediated immunity is of prime importance in mycobacterial infection (Kreeger, 1991).

Stabel (1996) found that peripheral blood mononuclear cells from subclinical paratuberculous cows produced significantly higher level of IFN-γ than cells from clinical animals after stimulation with mitogen Con A.

Sweeney *et al.* (1998) observed that IFN-γ expression in ileal tissue and caecal lymph nodes were significantly greater in cows with subclinical paratuberculosis, compared to cows with clinical disease.

Perez *et al.* (1999) while evaluating IFN-γ assay and intradermal skin test in sheep with naturally acquired paratuberculosis found that 55.4 percent sheep with pathologic changes had positive results in IFN-γ assay. Sensitivity and specificity of skin test, 55.6 per cent and 100 per cent, respectively were slightly higher. A close association between pathologic finding and cellular immune response was apparent. Most sheep with focal lesions and scant or no acid fast bacteria in the intestine had positive results of the IFN-γ assay and skin test. Most sheep with diffuse lesions and high number of bacilli were test negative.

Interferon-γ (IFN-γ) assay, which measures production of IFN-γ by leukocytes stimulated *in vitro* is superior to tests for humoral antibody in the detection of subclinical infection in sheep (Gwozdz *et al.*, 2000b).

Cytokine is produced by sensitized lymphocytes as a part of a cellular immune response to MAP infection. Based on sandwich immune enzyme assay protocols, this test can be used to compare the amount of IFN-γ produced by peripheral blood leukocytes in response to stimulation by MAP antigens (Manning and Collins, 2001).

Stabel and Whitlock (2001) opined that IFN-γ assay was a very sensitive diagnostic tool for detection of subclinical paratuberculosis in cattle and was useful to remove infected animal from the herd.

An experimental oral infection of goats with caprine isolate of *Mycobacterium avium* subsp *paratuberculosis*, produced cellular recall responses that were analysed by means of lymphocyte proliferation test, an IFN-γ assay and IL-2 receptor assay. All inoculated animals had detectable cell mediated immune responses from nine week post inoculation and the responses were highest during the first year (Storset *et al.*, 2001).

Jungersen *et al.* (2002) noted that IFN-γ test was the most appropriate tool for evaluation of disease preventive measures in young stock. The IFN-γ test could identify animals with possible sub clinical paratuberculosis infection before the animal became positive by faecal culture. Interferon-γ (IFN-γ) test was the only diagnostic test with a potential for diagnosing sub clinical paratuberculosis in live animal. While animals with

clinical paratuberculosis might exhibit low cellular immune response and thus present negative IFN-γ test results at stages of the disease at which they are positive by other tests.

It was not possible to predict from the IFN-γ test result, the number of animals that would eventually develop paratuberculosis. However the test might be useful to detect animals that had been exposed to MAP earlier in their lives and the testing of young cattle could be included in control programme to check for the effectiveness of preventing transmission of infection to calves and to identify animals at risk of developing disease later in their lives (Huda *et al.*, 2003).

Test measuring CMI could improve control of paratuberculosis, if able to diagnose mycobacterial infections earlier, before animal become infectious. Low specificity was reported as a draw back of CMI test for paratuberculosis. If the Johnin skin test and its *in vitro* equivalent, the IFN-γ assay were used in parallel and only animals positive on both test were considered as MAP infected, the specificity was 97.6 per cent. This support their potential value in the early diagnosis and control of paratuberculosis (Kalis *et al.*, 2003).

Storset *et al.* (2005) found that low response of non-infected goats could make the IFN-γ assay a useful tool in monitoring the paratuberculosis status of non vaccinated herds .

Cell mediated immune diagnostic test such as the IFN-γ assay and the Johnin skin test have the potential to detect animals infected with MAP early in the course of disease. While these CMI tests tend to be relatively specific in non-infected flocks, in MAP infected flocks these tests often identify animals that cannot be confirmed as infected by any other reference test, including necropsy and culture (Austerman *et al.*, 2007).

Study conducted by Lybeck *et al.* (2007) in Norwegian goat herds with naturally occurring paratuberculosis showed that ELISA and IFN-γ tests had the same ability to identify culture positive animals (about 80 per cent), while more culture negative goats were positive on IFN-γ testing, indicating that shedding could occur without any detectable immune response. The IFN-γ and ELISA testing of goats less than 18 months old rarely gave positive results. Some goats that were negative on IFN-γ test at 23 months of age became positive on ELISA one year later. As expected, IFN-γ test was the most sensitive

in detecting sub clinically infected animals. Some culture positive goats had only antibody response and no detectable IFN-γ response.

Interferon-γ assay is based on cellular immunity of host. This test measures the IFN-γ production of sensitised T cells by an immuno enzyme assay. Infected animals produce three fold more IFN-γ prior to shedding of the bacilli with faeces. In paratuberculosis, this test seemed to be more appropriate as a support tool to evaluate young animals of a herd (Lilenbaum *et al.*, 2007).

.2.6.2.3. Serological tests

In general, because CMI develops early in MAP infection and humoral immunity develops 10-17 months after infection, serological tests are not recommended for animals younger than 15 months (Nielsen *et al.*, 2001). The humoral response tends to occur relatively late in infection, those tests are better used for detection of clinical than sub clinical disease.

Serologic tests for *Mycobacterium avium* subsp *paratuberculosis* were most useful in establishing the herd prevalence of infection, for presumptive identification of infected animals and for confirming the diagnosis of Johne's disease in animals that demonstrates compatible clinical signs. A variety of tests can detect humoral antibodies to MAP in serum, but AGID, CFT and absorbed ELISA have been widely used to diagnose Johne's disease (NRC, 2003).

2.6.2.3.1. Complement fixation test (CFT)

Hole and Maclay (1959) reported that CFT was of no value in the very early stage of infection but had a marked practical value for the confirmation of suspected case.

Gilmour (1976) demonstrated that CFT was positive in about 90 per cent of the cattle with advanced disease.

Among 177 MAP infected cattle, Socket *et al.* (1992) demonstrated a test sensitivity of 38.4 per cent for CFT.

Sigurdardottir *et al.* (1999) recorded that CFT had been successfully employed in mapping disease in goat herds in Norway.

Complement fixation test (CFT) was one of the earliest serologic tests for Johne's disease. Compliment fixation test's major advantage is its ability to detect heavily infected animals. Most animals with a CFT titre of 1:32 or above are likely to be faecal culture positive (NRC, 2003).

Stewart *et al.* (2006) reported that serum antibody assays have diagnostic application in later stages of Johne's disease.

2.6.2.3.2. Agar gel immuno diffusion (AGID)

Sherman and Gezon (1980) suggested that serious consideration should be given to the immuno diffusion test as a diagnostic tool in control programs for caprine para-tuberculosis.

Kreeger (1991) suggested that a positive co-relation appeared to exist between faecal shedding of *Mycobacterium avium* subsp *paratuberculosis* and positive AGID results.

A test sensitivity of 26.6 per cent among 177 MAP infected cattle for agar gel immuno diffusion was observed by Socket *et al.* (1992).

Stehman (1996) reported that AGID was a highly specific serologic test for diagnosis of clinical paratuberculosis that correlated with faecal shedding of organism and degree of severity of lesion in small ruminants. However, it was less sensitive than faecal culture at the herd level for detection of subclinical disease.

Perez *et al.* (1997) noted a close relation between pathologic findings and serologic response, especially between presence of acid fast bacilli (in potentially heavily shedding

sheep) in the intestinal lesions and positivity to AGID. Agar gel immuno diffusion was proposed as a useful tool for seeking evidence of Johne's disease in live sheep.

For sero-diagnosis of Johne's disease in goats AGID test can be used (Goswami and Ram, 1998).

Munjal *et al.* (2005) observed that ELISA and AGID test were100 per cent effective in detecting experimentally infected paratuberculous goats from 180 and 210 days post infection onwards respectively.

Agar gel immuno diffusion was developed as a quick test for animals that were showing clinical signs of Johne's disease (Tiwari *et al.*, 2006).

Lilenbaum *et al.* (2007) observed that AGID could achieve a specificity of 100 per cent but was less sensitive than ELISA in detecting paratuberculosis in cattle.

2.6.2.3.3. Enzyme Linked Immuno Sorbent Assay (ELISA)

Kreeger (1991) opined that generally best use of ELISA was to rule out paratuberculosis infection.

Test sensitivity noted by Socket *et al.* (1992) for ELISA was 58.8 per cent during a study among 177 *Mycobacterium avium* subsp *paratuberculosis* infected cattle.

Sensitivity of ELISA was only 15 per cent in animals shedding low quantities of the *Mycobacterium avium* subsp *paratuberculosis* in their faeces, compared with 87 per cent in animals with clinical signs (Sweeney *et al.*, 1995).

Clarke *et al.* (1996) reported that ELISA and AGID tests were highly sensitive in diagnosis of multibacillary form of paratuberculosis in sheep and less sensitive in identifying animals with paucibacillary form of disease.

Out of 56 Johne's disease positive cattle ELISA could detect 43 animals (76 per cent) (Egan *et al.*, 1999).

For diagnosis of paratuberculosis in cattle, ELISA had a higher sensitivity in animals with a heavier load *ie,* high shedders (75 per cent) compared to low Shedders (15 per cent). Sensitivity of ELISA test decreased with repeated herd testing over time, since heavy shedders would be culled first from the herds (Whitlock *et al.,* 2000).

Jungersen *et al.* (2002) reported that *Mycobacterium phlei* absorbed ELISA was the most sensitive and specific assay available for diagnosis of Johne's disease, among the serological assays available for diagnosis

Vihan (2002) noticed that *Mycobacterium paratuberculosis* was known to share a broad range of cross reacting antigenic components with other species of mycobacterium thereby diminishing potential usefulness of ELISA.

Kostoulas *et al.* (2006) demonstrated that serum ELISA for paratuberculosis performed better in goats than in sheep.

In a study conducted by Rajkhowa *et al.* (2007), indirect ELISA detected maximum number of positive cases of paratuberculosis infection in Mithun, compared to Johnin and rectal pinch smear examination.

Enzyme linked immuno sorbent assay was more sensitive than CFT and AGID tests since they were capable of detecting antibody against *Mycobacterium paratuberculosis* at low concentration. Eighty per cent of the animals sero converted before appearance of clinical symptoms. Seventy per cent of the animals shed bacilli and 96 per cent could be identified by a combination of immunological assay and culture (Lilenbaum *et al.*, 2007).

2.6.3. Polymerase Chain Reaction (PCR)

A breakthrough in the diagnosis of Johne's disease was the discovery of IS*900*, a multi copy DNA insertion sequence unique to the genome of MAP (Collins *et al.,* 1989).

Amplification based on IS*900* has been developed and was widely used for the identification of *Mycobacterium avium* subsp *paratuberculosis* (Vary *et al.*, 1990).

Moss *et al.* (1991) reported that with PCR only small amount of DNA was required and that the purity of the sample was not always critical. Amplification could be performed with primer p 11 and p 36, based on IS*900* of MAP, resulting in a 279 bp PCR product.

Socket *et al.* (1992) noted that in PCR method, the gene technological detection of the insertion element IS*900* with a specificity of 100 per cent provided results as early as one or two days later.

Whipple *et al.* (1992) opined that generally the probe test detected MAP DNA in faecal specimen from animals shedding at least 10^4 MAP colony forming unit per gram of faeces. Although probe test did not detect all cattle shedding MAP, it was possible to identify cattle shedding the greatest number of organisms in three days, compared with a minimum of six weeks required for positive culture results.

Plante *et al.* (1996) concluded that PCR was a specific and relatively sensitive method for confirming Johne's disease.

Taejong *et al.* (1997) observed that PCR was much more rapid than conventional culture techniques for diagnosing Johne's disease.

Cousins *et al.* (1999) reported that the PCR targeting the 5' end of IS*900* had been considered specific for identification of MAP and was frequently applied to confirm the presence of *Mycobacterium avium* subsp *paratuberculosis* in the diagnosis of Johne's disease and recommended adoption of restriction endonuclease analysis of IS*900* PCR product as a routine precaution to prevent the reporting of false positive IS*900* PCR results.

Polymerase chain reaction was a powerful tool in diagnosing mycobacteral infections, although reduced sensitivity has been observed when amplification techniques has been used for analysis of clinical samples. The low sensitivity can be caused by the presence of inhibitors, the inefficient extraction of mycobacterial DNA and low number of bacteria present in the sample. Furthermore mycobacteria in samples from low shedders remain the most difficult to detect by PCR (Englund *et al.*, 1999).

Garrido *et al.* (2000) recorded that PCR technique was a promising alternative for specific determination of MAP in pathological samples within a shorter time.

Presence of *Mycobacterium paratuberculosis* could be detected in raw milk by PCR, but bacterial culture of milk was more sensitive (Giese and Ahrens, 2000).

Immuno magnetic separation PCR assay could detect approximately 10^3 colony forming unit (CFU) of *Mycobacterium paratuberculosis* per 50 ml of milk (equivalent to 20 CFU per ml), whereas the minimum detection limit of direct IS*900* PCR was estimated at 10^5 CFU of *Mycobacterium paratuberculosis* per 50 ml of milk (equivalent to 2000 CFU per ml) (Grant *et al.*, 2000).

Englund *et al.* (2001) opined that fluorescent PCR was a suitable alternative to both single PCR and nested PCR for the detection of MAP.

The advent of diagnostic probes based on specific bacterial sequences has allowed fastidious micro organisms such as MAP to be rapidly identified. Insertion sequence elements are small mobile genetic elements containing genes related to transposition functions (Harris and Barletta, 2001).

Marsh and Whittington (2001) found that direct PCR from faeces could be used as a rapid means of screening pooled faecal sample for flock diagnosis of Johne's disease in sheep.

The DNA of *Mycobacterium paratuberculosis* is > 99 per cent identical with that of *Mycobacterium avium*. The genetic feature that distinguishes one from the other is the presence of multiple copies of a short DNA element called an insertion sequence (IS) that is unique to MAP and is named IS*900* (Collins, 2003a).

Ibrahim *et al.* (2004) reported that molecular biological methods increased the sensitivity, specificity and rapidity of detection of MAP and PCR could be used successfully to detect *Mycobacterium avium* subsp *paratuberculosis* from crude bacterial DNA extract.

Vansnick *et al.* (2004) opined that the combination of two PCR assays (IS*900* PCR assay and F57 assay) with newly developed primers had proven to be superior in the identification of MAP.

Direct PCR based detection of MAP insertion sequence IS*900* from tissues and faeces had demonstrated that PCR was highly specific and sensitive. Polymerase chain reaction had been used positively to identify samples that were culture negative and could detect fentograms (less than 2 genome copies) amount of DNA (Huntley *et al.*, 2005).

Munjal *et al.* (2005) after experiential induction of infection in 5-8 weeks old kids observed that PCR could detect infected goat at 210 day post infection in faeces and 60 days post infection from tissue samples.

Polymerase chain reaction was found to have more sensitivity than bacterial culture and smear examination for diagnosis of Johne's disease (Sivakumar *et al.*, 2005).

Whitaker (2005) opined that PCR could be used in the future as a screening tool to eliminate subclinical shedders from the herd and reduce environmental contamination.

Mycobacterium avium subsp *paratuberculosis* genome is reported to have 15-20 copies of the insertion element. This high target copy number gives an increased sensitivity compared to systems targeting single copy genes, which makes it popular in molecular diagnostic method for paratuberculosis. Gene F57 is hither to considered specific for MAP and has no non-similarities in genes of other bacteria. However because it only appears once in the MAP genome, a PCR system based on F57 is expected to be less sensitive than the IS*900* systems (Herthnek, 2006).

Bhide *et al.* (2006) opined that disease control was hampered due to the hidden nature of Johne's disease and ineffective diagnosis, especially in the sub clinically infected animal population. The introduction of IS*900* dependent PCR has reduced the time and labour required for MAP diagnosis, owing to extremely slow progression of Johne's disease. Infected animal appear healthy without shedding MAP in milk or faeces, while harbouring potential infection in phagocytic cells such as macrophages. Such animals pose a real threat for the herd and with the help of IS*900* PCR technique MAP can be successfully detected from peripheral blood leucocytes of cattle and sheep.

Diagnostic tests based on specific DNA sequences of micro organisms allow fast and secure identification in slow growing bacilli. Regardless of the presence of other

contaminating micro organisms in faeces, molecular diagnosis specifically detects MAP. It was suggested that at least 10,000 bacilli per gram of faeces were required to achieve positive result in PCR. One of the possible reasons for this low sensitivity is the presence of inhibiting substances in faeces and the difficulty to remove them (Lilenbaum *et al.*, 2007).

United States Department of Agriculture (USDA) regulations stated that an organism based test (PCR or culture) is the official test for determining the infective status of an animal for Johne's disease (Payeur and Caspel, 2007).

2.7. CONTROL

According to Amstutz (1984) measures which could be taken in herds to reduce losses included raising replacement animals separate from adults, slaughtering of animals shedding MAP in their faeces and decontaminating the premises.

The first phase of control is to identify infected animals. The insidious nature of the infection, the long incubation period and the slow development of lesions makes the disease one of the most difficult to recognize and control. The failure of diagnostic methods to identify all infected animals hinders control programs. To avoid Johne's disease, animals must only be purchased from well established herds which are certified paratuberculosis free. All the adult animals in the herd should be tested by faecal culture every six months and all the positive animals and their latest offspring should be sent immediately to slaughter regardless of condition or productivity. Such semi annual testing and culling will eliminate infected animals before clinical illness and eliminate those animals most likely to become infected. Calves should be removed from the dam before they nurse and be placed in an area which has not been contaminated by faeces. Care must be taken in obtaining colostrum for these calves. Udders must be washed with a disinfectant prior to milking. Natural nursing must not be allowed. Disposal of infectious faeces creates special consideration. Liquid processing of faeces and disinfection by addition of twenty percent

hydrated lime produces lime stabilization of faeces and destroy the bacillus.(Chiodini *et al.*, 1984).

Rossiter and Burhans (1996) opined that individual plan should be developed and adapted to individual farm circumstances for effective control of Johne's disease.

Effective disease control programmes depend on addressing potential source of infection and routes of transmission. Studies have demonstrated that MAP is viable for up to 250 days in water, faeces and cattle slurry. Consequently contamination of an animal's environment by manure from infected animals is the most common mode of transmission. Vertical transmission during pregnancy has also been implicated since MAP has been isolated from the uterus, foetal tissues and semen (Harris and Barletta, 2001).

Early detection and removal of massive sources of infection as well as effective cleaning and disinfection of the stable environment and pastures play a paramount role in successful control of paratuberculosis. Ten per cent of subclinically infected cows and fifty per cent of animals showing clinical paratuberculosis excreted the organism in the milk (Pavlas, 2005).

Culling of faecal shedding cows might help to reduce the environmental burden of MAP. Reducing the quantity of MAP being shed by cows with Johne's disease should decrease the risk of spread of this disease to young stock (Hendrick *et al.*, 2006).

Raizman *et al.* (2006) opined that current diagnostic tests lack the desired sensitivity to detect subclinically infected cattle, individual animals intended as herd replacements from infected herds cannot be effectively screened prior to introduction to a herd. The recommended on farm control measures include identification and removal of infected animal from the herd to prevent further transmission. Prevention of calf ingestion of the organism in adult cow manure, milk, colostrum and water and reduction of environmental contamination to reduce overall exposure to the organism.

Bacteria might be excreted in faeces by animals for up to eighteen months before clinical signs are apparent, but shedding is particularly high (up to 5×10^{12} mycobacteria

per day) from clinical cases. So proper disposal of dung should be undertaken since it is the main source of infection (Nandi, 2008).

2.7.1. Vaccination

Vaccination of cattle against Johne's disease had been widely practised in France since the development of vaccine by Vallee and Rinjard in 1926, which consisted of a suspension of living *Mycobacterium johnei* in a mixture of olive oil and liquid paraffin to which traces of fine pumice powder was added. In 1945 Doyle was the first to use French type of vaccine in Britain (Spears, 1959).

Vaccination with an adjuvanated vaccine containing live or killed strain of *Mycobacterium johnei* gave protection against clinical Johne's disease. Vaccination did not eliminate infection from a herd but it reduced the faecal excretion of organisms (Gilmour 1976).

Moser (1982) observed that vaccination apparently did not prevent infection but did reduce the incidence of clinical cases and shedding of organism in goat.

Chiodini *et al.* (1984) reported that sub cutaneous inoculation of either killed or live vaccine suspended in mineral oil was capable of reducing the number of animals that developed clinical diseases with excretion of *Mycobacterium avium* subsp *para-tuberculosis*.

Benedictus *et al.* (2000) noted that vaccination of young animals seemed to be an effective measure in prevention of clinical paratuberculosis, although changes in management and hygiene practices were also important. In Iceland, successful eradication of paratuberculosis was achieved by vaccinating the lambs once with a killed vaccine and containment of sheep within one specific region during the eradication campaign. In Norway after several years of unsuccessful efforts to eradicate Johne's disease in goat herds by general hygienic methods and the isolation and slaughter of clinically affected animals, a vaccination programme with an attenuated live vaccine for kids aged 2-4 weeks

was started in 1967. Based on post mortem data the infection with MAP was reduced from fifty three to one per cent. In Netherlands from 1983 a programme focussed on vaccination of calves with a killed vaccine was started and this strategy had been successful in reducing clinical paratuberculosis

Corpa *et al.* (2000) reported that vaccination with killed MAP vaccine had been widely used as a measure for controlling paratuberculosis.

Extensive research by Dr.Bjorn Sigurdsson and co-workers led to the development of a paratuberculosis vaccine that is still used in Iceland. The Icelandic vaccine or the so called Sigurdsson's vaccine consists of two heat killed bovine strains of the bacteria suspended in equal parts of olive oil and mineral oil. Vaccination experiment began in sheep in 1950 and showed that vaccination could reduce mortality in sheep by about 90 per cent. Vaccination of sheep in endemic area has been compulsory in Iceland since 1966 and as a result losses have become reduced considerably. Today, infection is kept under control by vaccinating breeding lambs in endemic areas at the age of 4-6 months in the autumn (Fridriksdottir *et al.*, 2000).

Vaccination of lambs with live attenuated vaccine two weeks after oral inoculation with MAP, stimulated the host response against the organism and led to a reduced mycobacterial burden (Gwozdz *et al.*, 2000a).

Uzonna *et al.* (2003) opined that efficacy of paratuberculosis vaccine might be enhanced if calves were vaccinated prior to *Mycobacterium paratuberculosis* exposure with field isolate vaccine instead of strain 18 vaccine currently used in bovine.

2.8. ZOONOTIC POTENTIAL

Chiodini *et al.* (1984) opined that similarities in the clinical and histological features between bovine paratuberculosis, intestinal tuberculosis and Crohn's disease and recovery of *Mycobacterium avium* subsp *paratuberculosis* from intestinal tissues of patients with

Crohn's disease have encouraged researchers to investigate the possibility of a *mycobacterial* etiology in Crohn's disease.

Intact cell wall deficient (spheroplast) *mycobacterial* agent resembling in many respects with *Mycobacterium avium* subsp *paratuberculosis* has been isolated from several cases of Crohn's disease (Kreeger 1991).

Ever since its first description by Dalziel and rediscovery by others, successive generation of clinicians have speculated about a *mycobacterial* cause for what is now known as Crohn's disease. The idea has been supported by reports of *Mycobacterium avium* subsp *paratuberculosis* detected in tissues of patients with Crohn's disease by culture and by molecular methods (~60 per cent and ~80 per cent respectively). Recent data has strengthened the association of MAP with Crohn's disease. The reactivity of anti *Mycobacterium paratuberculosis* antibodies (specifically against p35 and p36 antigens) in a significant proportion of Crohn's disease patients would suggest a causal role for the organism in Crohn's disease (Naser *et al.*, 2000).

Collins (2003a) reported the thermal tolerance of MAP, specifically the capacity to survive pasteurization and it is a subject of considerable interest.

The clinical and pathological resemblance of Crohn's disease and Johne's disease was recognized when this chronic inflammatory bowel disease was first recognized in humans in 1913, just eighteen years after Johne's disease was first reported. Clinically diarrhoea and weight loss are the predominant signs in both diseases. Abdominal pain and fever are the part of the constellation of signs in Crohn's disease but they appear to be absent in Johne's disease. The triggering event of Crohn's disease is thought to occur early in life and then be followed by 15-30 years of incubation or latency period. Johne's disease also has a long interval between infection with MAP and onset of clinical signs (2-10 years). In both diseases clinical signs are seldom seen before sexual maturity. The first reported isolation of *Mycobacterium avium* subsp *paratuberculosis* from Crohn's disease patient was in 1984 and when IS*900* PCR was used, genetic material from MAP was detected in resected bowel tissues from Crohn's disease, more consistently than the living

organism was isolated by culture. This suggest an association between MAP and Crohn's disease (Collins, 2004).

Recently the organism was reported to be associated with enteric infection in humans and hence the disease is of public health importance (Ibrahim *et al.*,2004)

Lilenbaum *et al.* (2007) reported that thirty five percent of all animals presenting symptoms of Johne's disease and ten percent of animals in the subclinical phase shed bacillus in the milk. Since *Mycobacterium avium* subsp *paratuberculosis* organism can resist pasteurization, it can potentially contaminate human population.

There are several histopathological and clinical similarities between Johne's disease and Crohn's disease. Because of these similarities a *mycobacteral* cause of Crohn's disease has been sought for more than 90 years. Recent serological studies have demonstrated that up to eighty three percent of Crohn's disease patients showed evidence of serum antibodies to MAP. Cows with clinical paratuberculosis do shed viable organisms in their milk at low levels (50 CFU per 50 ml of milk) and the consumption of in adequately pasteurized dairy products has been a major source of concern for the potential spread of MAP to humans. As a conclusion it is suggested that solid evidence insinuate an association between Crohn's disease and *Mycobacterium avium* subsp *paratuberculosis* (Raizman, 2007).

Materials and methods

3. MATERIALS AND METHODS

The present study was carried out in the department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy, Trichur during 2007-2008.

3.1 MATERIALS

3.1.1 Experimental animals

Two hundred goats above six months of age of either sex from Kerala Agricultural University Sheep and Goat Farm (USGF) Mannuthy,Trichur and crossbred Malabari goats from field (Tanoor panchayath of Malappuram district) were used for the present study. The animals of USGF Mannuthy were born and reared in the farm and were showing no apparent clinical symptoms of paratuberculosis. Any animal showing signs of disease, as well as animals in late gestation were not included for the study. This criteria was applicable to animals from field and animals below six months of age was not included for the study. All the goats were maintained under standard feeding and management conditions.

3.1.2 Clinical material

Faecal samples collected from rectum of one hundred and fifty goats of USGF Mannuthy and fifty goats from field formed samples of study.

3.1.3 Glassware, plasticware and chemicals

All glassware used was of Borosil brand. Plastic ware used were of Tarfon brand and chemicals were of molecular biological grade.

3.1.4 Acid Fast Staining

3.1.4.1 Stains

3.1.4.1.1 Ziehl – Neelsen's carbol fuchsin solution

Basic Fuchsin	2 g
Crystalline phenol	10 g
Absolute alcohol	20 ml
Distilled water	200 ml

Basic Fuchsin was dissolved in phenol by placing in a 500 ml beaker over boiling water bath for five minutes. The contents were shaken frequently. When it was completely dissolved alcohol was added and mixed thoroughly. Then distilled water was added and sieved through a muslin cloth. It was kept in a glass stoppered bottle for a week.

3.1.4.1.2 Loeffler's Methylene blue

Saturated solution of Methylene Blue in alcohol	30 ml
KOH	10 mg
Distilled water	100 ml

Potassium hydroxide was dissolved in water and saturated solution of methylene blue in alcohol was added, mixed well, filtered through muslin cloth and stored in a glass stoppered bottle.

3.1.4.2.3 Decolourizing agent - 3% Acid alcohol

Hydrochloric acid (35.4%)	3 ml
Ethyl alcohol (95%)	97 ml

Seven ml of 95 per cent ethyl alcohol was added to three ml of acid and volume was made up to 100 ml by using ethyl alcohol.

3.1.5 Single intradermal test

3.1.5.1 Biologicals

3.1.5.1.1 Johnin

Johnin purified protein derivative (PPD) obtained from Indian Veterinary Research Institute (IVRI) , Izatnagar, UP was used for single intradermal test (Plate 1). Johnin PPD is a preparation of the heat treated products of growth and lysis of *Mycobacterium avium* subsp *paratuberculosis.* It contains 1 mg PPD per ml and preserved with 0.5 per cent phenol.

3.1.5.1.2 70% alcohol

3.1.5.2 Equipments

3.1.5..2.1 Vernier caliper

3.1.5..2.2 Tuberculin syringe

3.1.6 Polymerase chain reaction

3.1.6.1 PCR Primers (IDT TECHNOLOGIES, USA)

Standard PCR- Sequence of Primer

ORGANISM	*Mycobacterium avium* subsp *paratuberculosis*
GENETIC ELEMENT	IS*900*
PRIMER P36 F	5'-GGCCGTCGCTTAGGCTTCGA-3'
PRIMER P11 R	5'-CGTCGTTAATAACCATGCAG-3'
SIZE	279 bp
REFERENCE	Halldorsdottir *et al.* (2002)

3.1.6.2 2X Red Dye Master mix, (GENEI, BANGALORE)

3.1.6.3 DNA Molecular Weight Marker (GENEI LABORATORIES, BANGALORE)

100 bp ladder was used as the standard molecular weight marker

3.1.6.4 Agarose (GIBCO, USA)

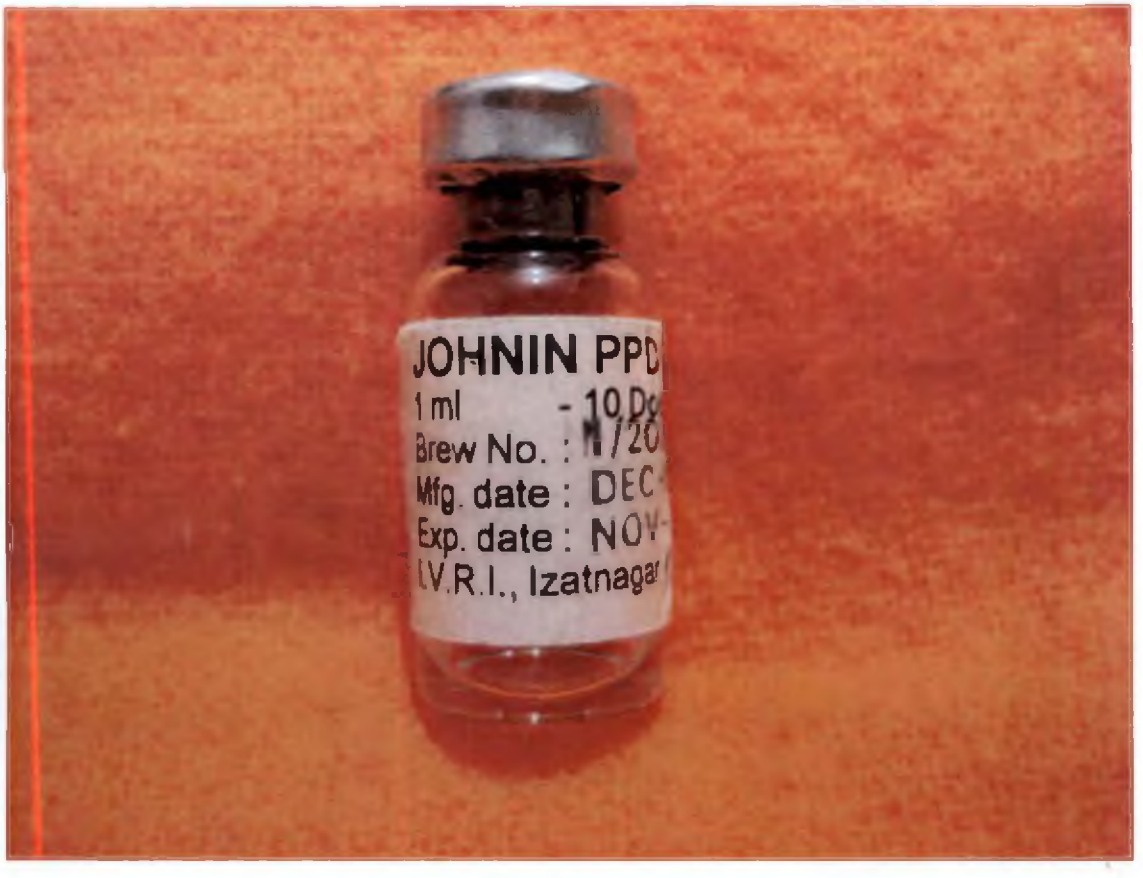

Plate 1. Johnin Purified Protein Derivative (PPD)

3.1.6.5 Reagents

3.1.6.5.1 Tris EDTA Buffer

1M Tris pH 7.4	10 ml
0.5M EDTA pH 8	2 ml
Water to make up to	1000 ml

3.1.6.5.2 Tris Acetate Gel running Buffer (TAE) Stock Solution 50X

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5mM EDTA (pH 8)	100.0 ml

The pH was adjusted to 8.2 with 0.1 N NaoH and the volume was made up to one litre with distilled water.

Working solution of TAE buffer (1X) –pH 8.2

It was prepared by mixing 20 ml of 50X TAE with 980 ml of triple distilled water.

3.1.6.5.3 Ethidium Bromide (SIGMA, USA) Stock solution

Ethidium bromide	25 mg
TAE Buffer	10 ml

Prepared solution was stored in a dark bottle at 4°C

3.1.6.5.4 Gel loading dye (6X)

Bromphenol blue	0.25%
Xylene Cyanol	0.25%
Ficoll(type400)	25% in distilled water

3.1.6.6. QIAamp DNA Stool Mini Kit (USA) (Plate 2)

Kit contents

QIAamp spin columns	50
Collection tubes (2ml)	200
InhibitEX tablets	50
Buffer ASL	140 ml
Buffer AL	33 ml
Buffer AW1 (concentrate)	19 ml
Buffer AW2 (concentrate)	13 ml
Buffer AE	12 ml
Proteinase K	1.4 ml

3.1.6.7 Positive control MAP Vaccine obtained from Iceland-"BOLUEFNI GEGNGARNAVEIKI"

1X PBS 500 µl was added to 100 µl vaccine. Centrifuged at 10000 x *g* for 10 minutes. Discarded the supernatant and to the pellet added 100 µl water. Vortexed and from this taken 2 µl, boiled for 5 minutes at 100°C and then snap cooled in ice and used as positive control

3.1.6.8 Ethanol 96-100%

3.1.6.9. Equipments

3.1.6.9.1 PCR Machine – Thermal Cycler (EPPENDORF ,USA)

3.1.6.9.2 Centrifuge (EPPENDORF, USA)

3.1.6.9.3 U V Transilluminator (FOTODYNE, USA)

3.1.6.9.4 Submarine Gel Electrophoresis Apparatus

Plate 2 . QIAamp DNA Stool Mini Kit

COMPONENTS

1 . Buffer AW1
2 . Buffer AW2
3 . Buffer AL
4 . Proteinase K
5 . Buffer AE
6 . Buffer ASL
7 . Inhibit EX tablets
8 . QIAamp spin columns

3.2 METHODS

3.2.1 Ziehl Neelsen's (Acid fast) Staining

Faecal smears were examined for *Mycobacterium avium* subsp *paratuberculosis* by acid fast staining as per Paliwal *et al.* (1984) with slight modification. Faecal samples of all the two hundred animals were directly collected from the rectum. Two to three pellets were mixed well with 10 ml of normal saline in a mortar and pestle. It was then sieved into a test tube and the suspension was kept undisturbed overnight. Supernatant was collected into a sterile test tube in the morning and centrifuged at 2000 rpm for five minutes. Poured off the supernatant and sediment of each sample was resuspended in one ml of normal saline and thick smears of these were made on a clean grease free glass slide, dried in air and heat fixed and examined for *Mycobacterium avium* subsp *paratuberculosis* by Ziehl-Neelsen technique. Presence of typical clumps of acid fast bacilli were taken as positive.

3.2.2 Single Intradermal Test

The single intradermal test was performed as per the method described by the OIE (2004). Site of test was the middle third of neck, approximately mid way between its upper and lower borders (Plate 3). An area of about one square inch was shaved using a sterile blade. The shaved area was mopped with 70 per cent alcohol. A fold of skin was picked up between the forefinger and thumb of one hand and measured with the calipers held in the other hand (Plate 4). Measurement of skin thickness was arrived at by taking three consecutive readings for each measurement and taking the average value into account. Thickness of fold of skin was recorded in millimeters.

A fold of skin in the middle of the area was held using left fore finger and thumb. The tuberculin needle was inserted parallel to the skin, with care taken to ensure that it did not pierce into deeper layers. Johnin 0.1 ml was injected intradermally (Plate 5). Accuracy of the technique was ascertained by the necessity

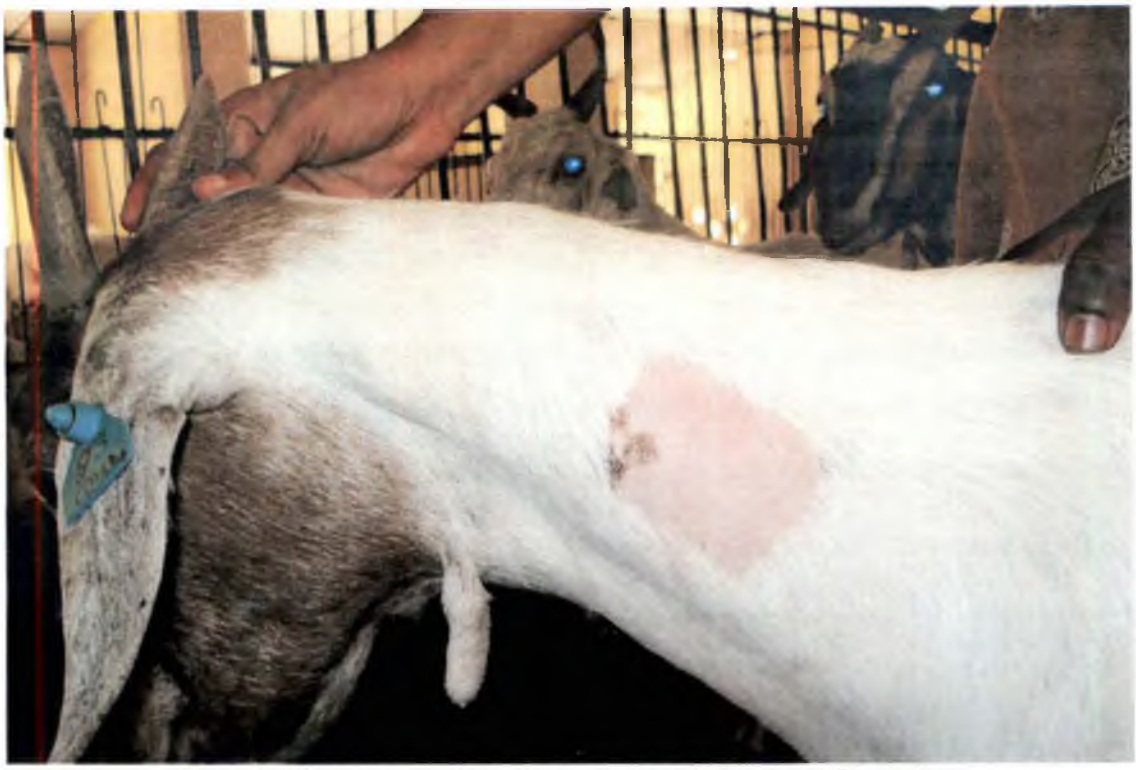

Plate 3. Site prepared for intradermal injection of Johnin

Plate 4. Measurement of skin thickness prior to intradermal injection

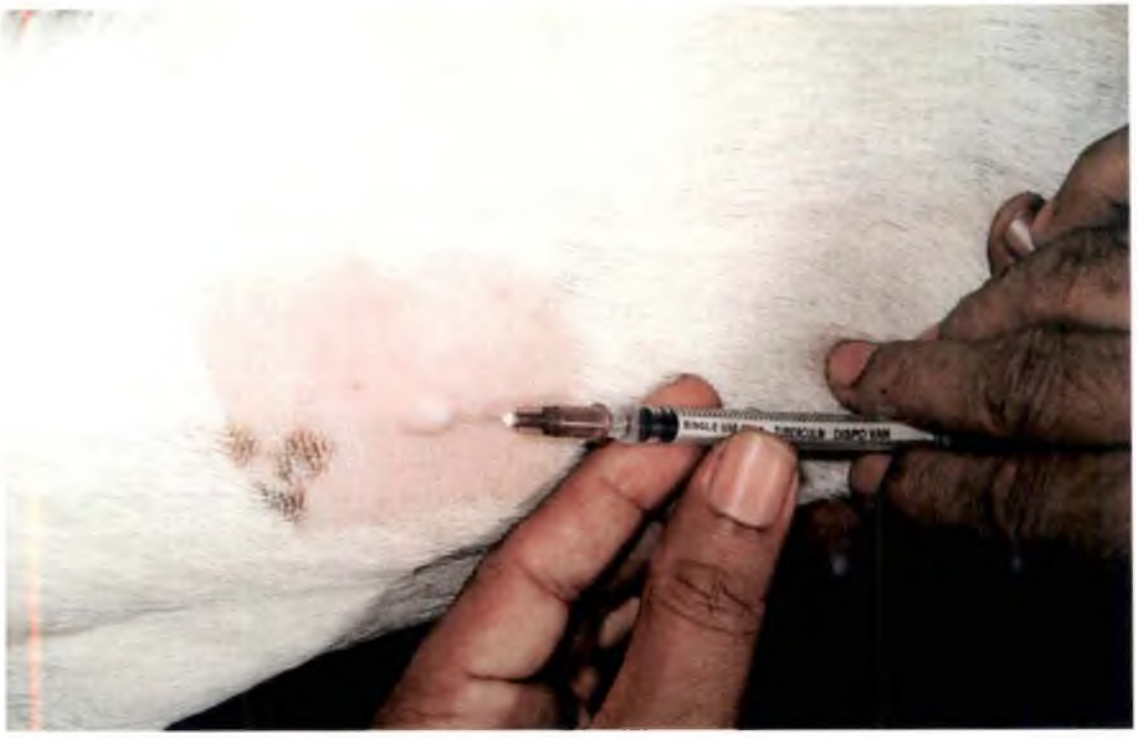

Plate 5. Intradermal inoculation of Johnin

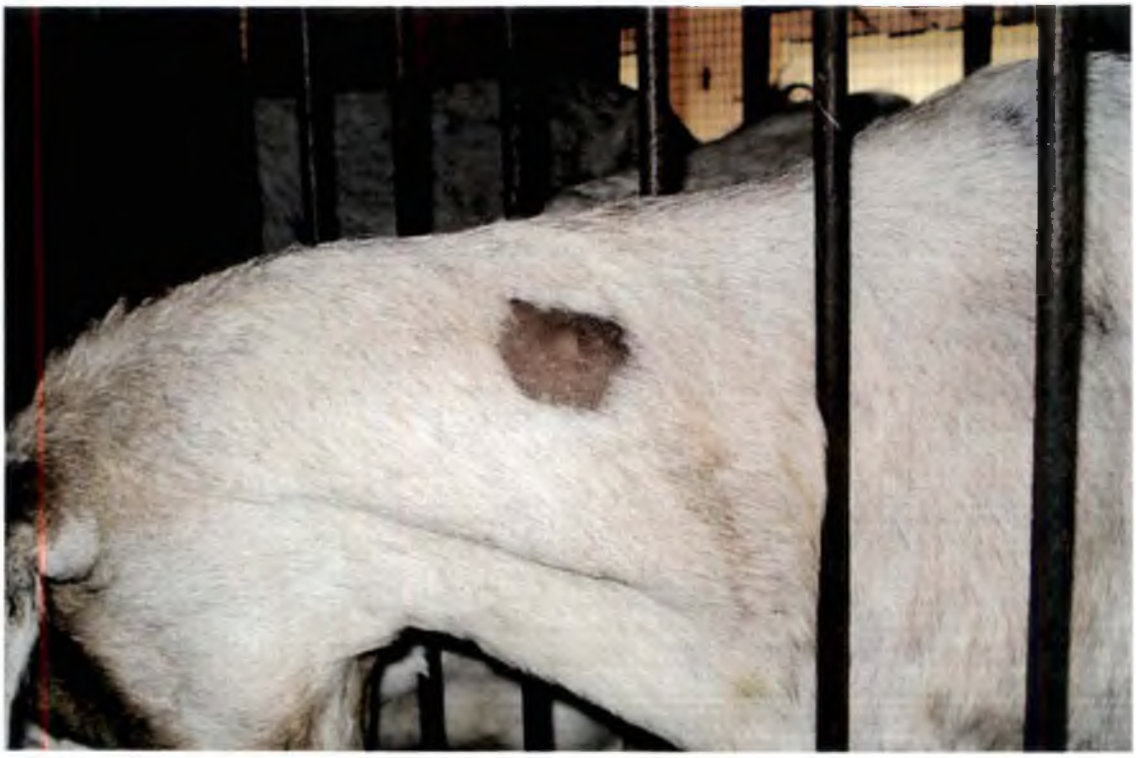

Plate 6. Development of pea-shaped nodule at the site of intradermal injection of Johnin

of very high pressure for injection and the development of a pea like nodule at the site (Plate 6). Seventy two hours after the injection read the reaction.

Interpretation-A positive reaction was recorded as an increase in thickness of four mm and above and the presence of a hot, painful and oedematous swelling at the site of injection.

3.2.3 Slaughter of animals and collection of materials

To investigate systematic pathological changes in single intradermal positive animals, three goats which gave positive results in single intradermal Johnin test from farm were slaughtered. Complete digestive tract and other visceral organs were macroscopically examined. Pieces of intestine were collected from ileum, ileocaecal valve, jejunum, caecum, colon and rectum and subjected to detailed gross examination. Ileocaecal and mesenteric lymph node were collected, impression smear from cut surface of lymph nodes were stained with Ziehl-Neelsen carbol fuchsin to detect acid fast bacilli.

3.2.4 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was carried out with Red Dye Master Mix (Bangalore Genei). The PCR assay was carried out as per the method of Halldorsdottir *et al.* (2002) with minor modifications.

Deoxyribonucleic acid was isolated from faecal sample using QIAamp DNA Stool Mini Kit.

Vaccine against *Mycobacterium avium* subsp *paratuberculosis* from Iceland was used as positive control.

3.2.4.1 Isolation of DNA from stool for detection of Mycobacterium avium subsp *paratuberculosis*

(1) 180-220 mg of stool was weighed in a two ml microcentrifuge tube and the tube was placed on ice.

(2) 1.4 ml Buffer ASL was added to each stool sample and was vortexed continuously for one minute until the stool sample was thoroughly homogenized.

(3) The suspension was heated for five minutes at 70°C.

(4) It was vortexed for fifteen seconds and the sample was centrifuged at 10000 x *g* for one minute to pellet stool particles.

(5) 1.2 ml of supernatant was pipetted into a new two ml microcentrifuge tube and the pellet was discarded.

(6) One InhibitEX tablet was added to each sample and vortexed immediately and continuously for one minute until the tablet was completely suspended. The suspension was incubated for one minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

(7) Sample was centrifuged at 10000 x *g* for three minutes to pellet stool particles and inhibitors bound to Inhibit EX.

(8) All the supernatant was pipetted in to a new 1.5 ml microcentrifuge tube and the pellet was discarded. The sample was centrifuged at 10000 x *g* for three minutes.

(9) 15µl of Proteinase K was pipetted into a new 1.5 ml microcentrifuge tube.

(10) 200 µl of supernatant was pipetted into 1.5 ml micro centrifuge tube containing Proteinase K.

(11) 200 µl of Buffer AL was added and vortexed for fifteen seconds.

(12) Incubated at 70°C for ten minutes.

(13) 200 µl of ethanol (96 to 100 per cent) was added to the lysate and mixed by vortexing.

(14) Labelled the lid of a new QIAamp spin column placed in a two ml collection tube. Carefully applied the complete lysate to the QIAamp spin column without moistening the rim. Closed the cap and centrifuged at 10000 x *g* for one minute. Placed the QIAamp spin column in a new two ml collection tube and discarded the tube containing the filtrate.

(15) The QIAamp spin column was opened carefully and 500 µl Buffer AW1 was added and centrifuged at 10000 x *g* for one minute. The QIAamp spin column was placed in a new two ml collection tube and the collection tube containing the filtrate was discarded.

(16) Carefully opened the QIAamp spin column and added 500 µl Buffer AW2. Centrifuged at 10000 x *g* for three minutes and discarded the collection tube containing the filtrate.

(17) The QIAamp spin column was transferred into a new labeled 1.5 ml micro centrifuge tube and 40 µl Buffer AE was pipetted directly onto the QIAamp membrane and incubated for 1 minute at room temperature and then centrifuged at 10000 x *g* for 1 minute to elute DNA.

3.2.4.2 Detection of MAP by standard PCR using the Primers for IS900 gene

Polymerase chain reaction assay was performed for the gene IS*900* using the primers for IS*900*. The reaction was performed using PCR Red dye master mix (2X) in 25 µl reaction volume in the following manner.

PCR master mix	12.5 µl
Primer F (50 picomol)	0.5 µl
Primer R (50 picomol)	0.5 µl
Template DNA	2.0 µl
Distilled water	9.5 µl

3.2.4.2.a PCR amplification condition

Amplification was carried out in a thermal cycler (Pate 7) using the following programme (Plate 8).

Step 1 Initial denaturation	94°C for 5 minutes
Step 2 Denaturation	94°C for 1 minute----} 35 cycles
Annealing	55°C for 1 minute----} 35 cycles
Extension	72°C for 1 minute----} 35 cycles
Step 3 Final extension	72°C for 7 minutes

3.2.4.3 Detection of PCR products –Agarose Gel Electrophoresis

Two percent agarose gel was prepared with 1X TAE buffer. A clean dry gel platform was selected and the sides were sealed using good quality adhesive tapes. The tray was placed on a levelled surface and suitable combs intended for preparation of wells were placed in the plat form. Two gram agarose in 100 ml TAE buffer was heated in a microwave oven until it was a clear solution, cooled to 60 °C and 20 µl of ethidium bromide was added and mixed well. The mixture was cooled

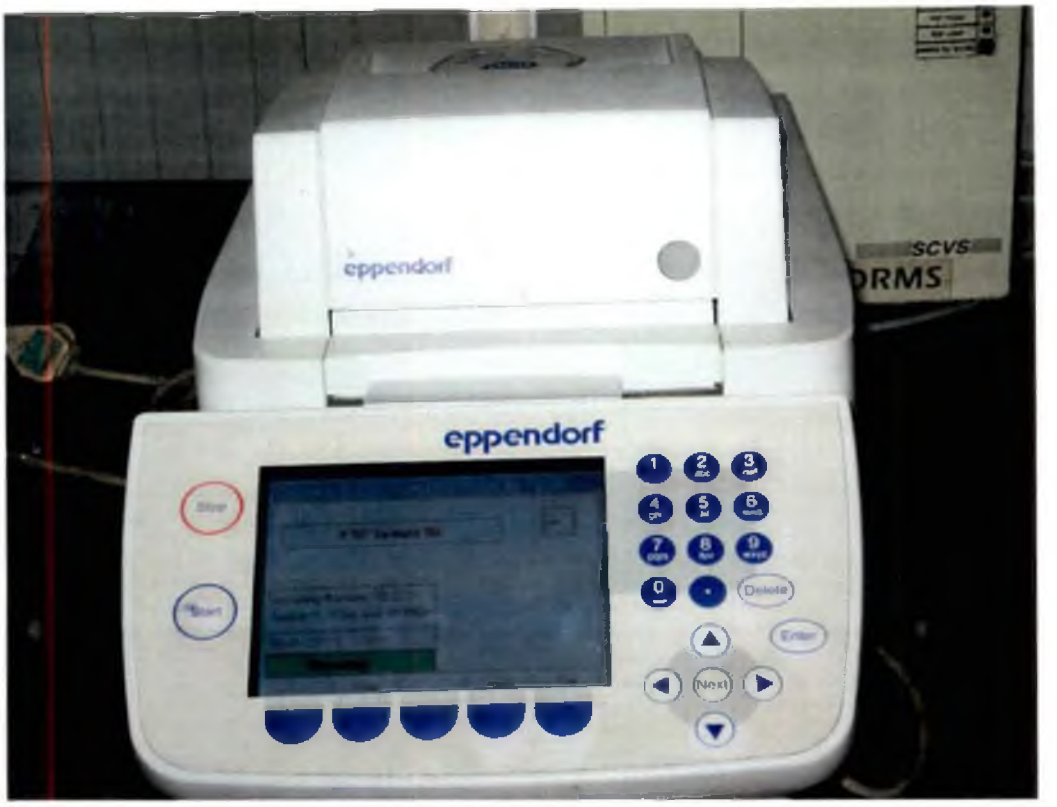

Plate 7. Eppendorf thermal cycler

Plate 8. PCR program for DNA amplification running

to hand bearable temperature and carefully poured into the gel tray avoiding air bubbles so as to make a gel of five mm thickness. Once the gel got solidified, the combs were taken out and then the wells could be appreciated.

The samples were loaded along with the DNA markers and the gel was run at 100 volts for 30 minutes (Plate 9) to separate the IS*900* PCR products by electrophoresis. The amplification was visualised by ultraviolet trans-illumination and was documented using gel documentation system. Appearance of a band at 279 bp when compared with the DNA ladder was taken as positive.

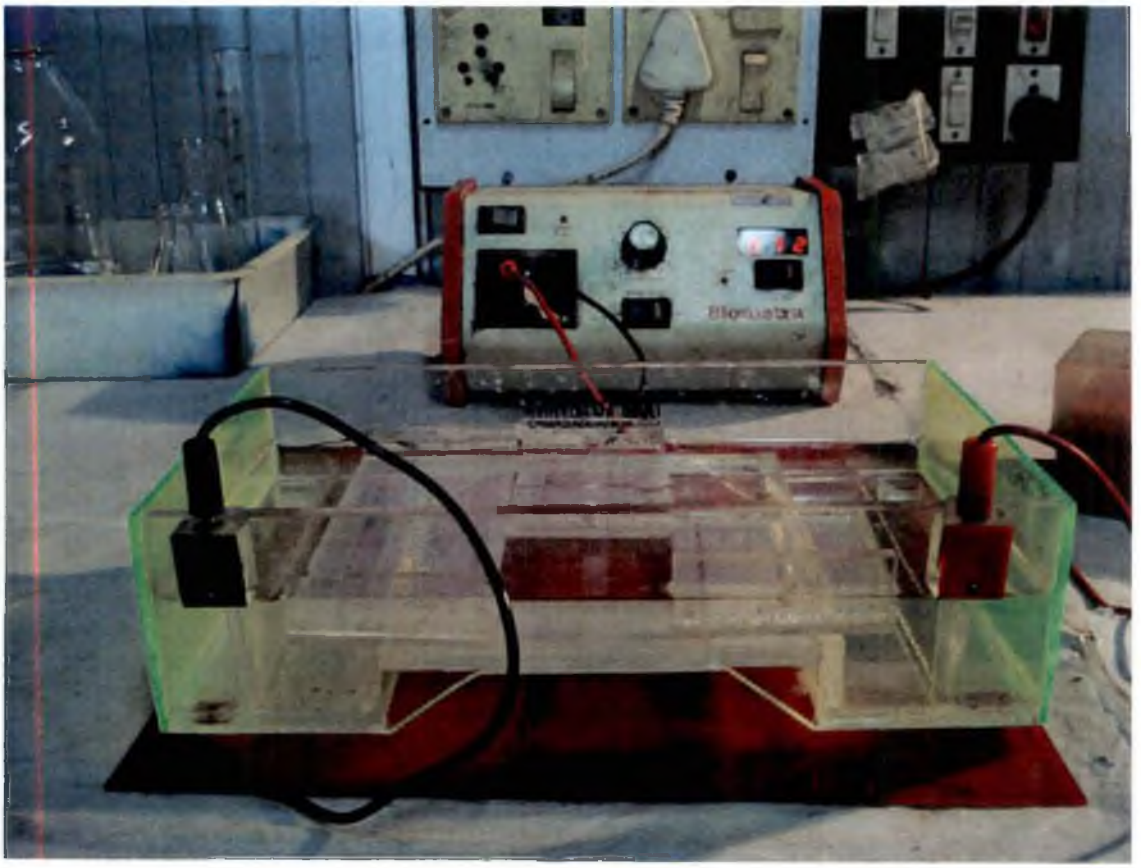

Plate 9. Submarine agarose gel electrophoresis

Results

4.RESULTS

4.1 COMPARATIVE EFFICACY OF VARIOUS DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS

Comparative efficacy of three diagnostic tests *viz.*, Ziehl- Neelsen acid fast staining, single intradermal Johnin test and polymerase chain reaction IS*900* in goats were carried out in the present study and the results are presented (table 1to 7 and figures 1 to 16).

4.1.1 Microscopic faecal smear examination by Ziehl-Neelsen's acid fast staining

4.1.1.1 Results from University sheep and goat farm (USGF) Mannuthy

A total of one hundred and fifty goats from USGF, Mannuthy were screened for paratuberculosis by faecal smear examination by Ziehl-Neelsen acid fast staining. The results are presented in Table (1). Out of one hundred and fifty animals screened only five animals were found positive (Plate 10) by this test, giving a test positivity per centage of 3.3.

4.1.1.2 Results from field

Fifty goats from field were screened for Johne's disease by acid fast staining of faecal smear. Results are given in Table (1). Only one animal from field was found positive by this test for paratuberculosis, giving a test positivity per centage of two.

4.1.2 Single intradermal Johnin test

4.1.2.1 Results from USGF Mannuthy

The concised results of single intradermal Johnin test for farm are given in Table (2). Eight of the one hundred and fifty animals tested had positive reactions (Plate 11).

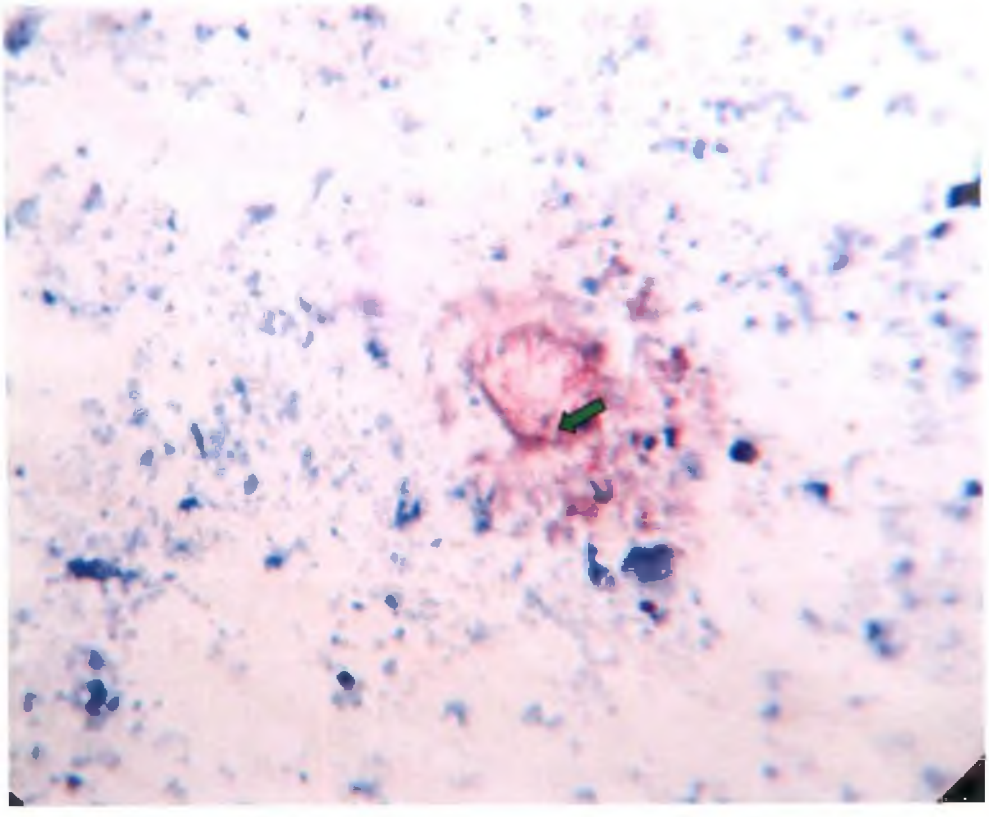

Plate 10. Clumps of acid fast organism in faecal smear

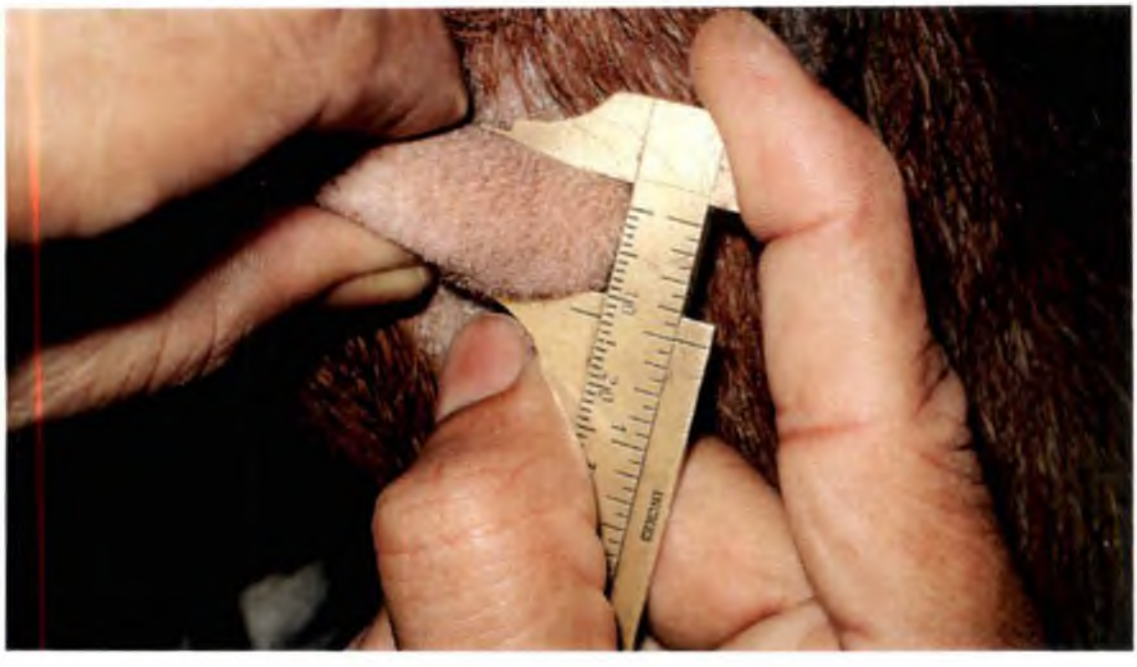

Plate 11. Single intradermal test showing edematous swelling is measured by vernier caliper (72 hours after injection)

4.1.2.2 Results from field

Out of fifty animals tested for paratuberculosis by single intradermal Johnin test three were found positive and the results are presented in Table (2).

4.1.3 Post mortem findings

Three animals were randomly selected for slaughter based on the positive result of single intradermal Johnin test from farm and the findings are given in Table (7). All the three animals were infected with *Mycobacterium avium* subsp *paratuberculosis*. In animal with oedematous and enlarged lymph node no acid fast bacteria could be detected (Plate 12). Animal with corrugation in ileum revealed acid fast bacteria in lymph node (Plate 13). One animal had no gross lesion but on microscopical examination of lymph node impression smear showed presence of typical clumps of acid fast bacteria (Plate 14).

4.1.4 Polymerase Chain Reaction IS*900*

4.1.4.1 Results from USGF Mannuthy

Number of goats detected positive for paratuberculosis by PCR IS*900* are presented in Table (3). Among the one hundred and fifty animals tested thirty were found positive (Plate 15) for *Mycobacterium avium* subsp *paratuberculosis* (20 per cent).

4.1.4.2 Results from field

Test result of IS *900* PCR for field animals are given in Table (3). Out of fifty animals tested twelve animals (24 per cent) were found positive for Johne's disease by this test.

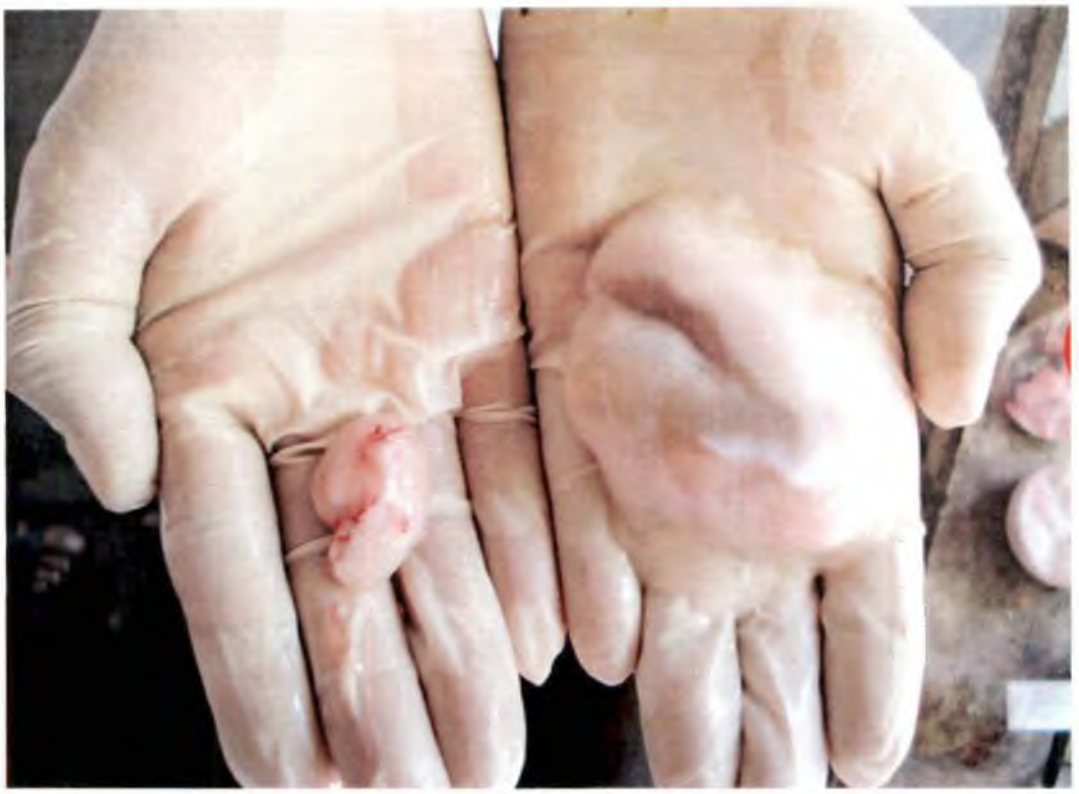

Plate 12. Enlarged edematous mesenteric lymph node from Johne's disease positive goat (right) and normal specimen (left)

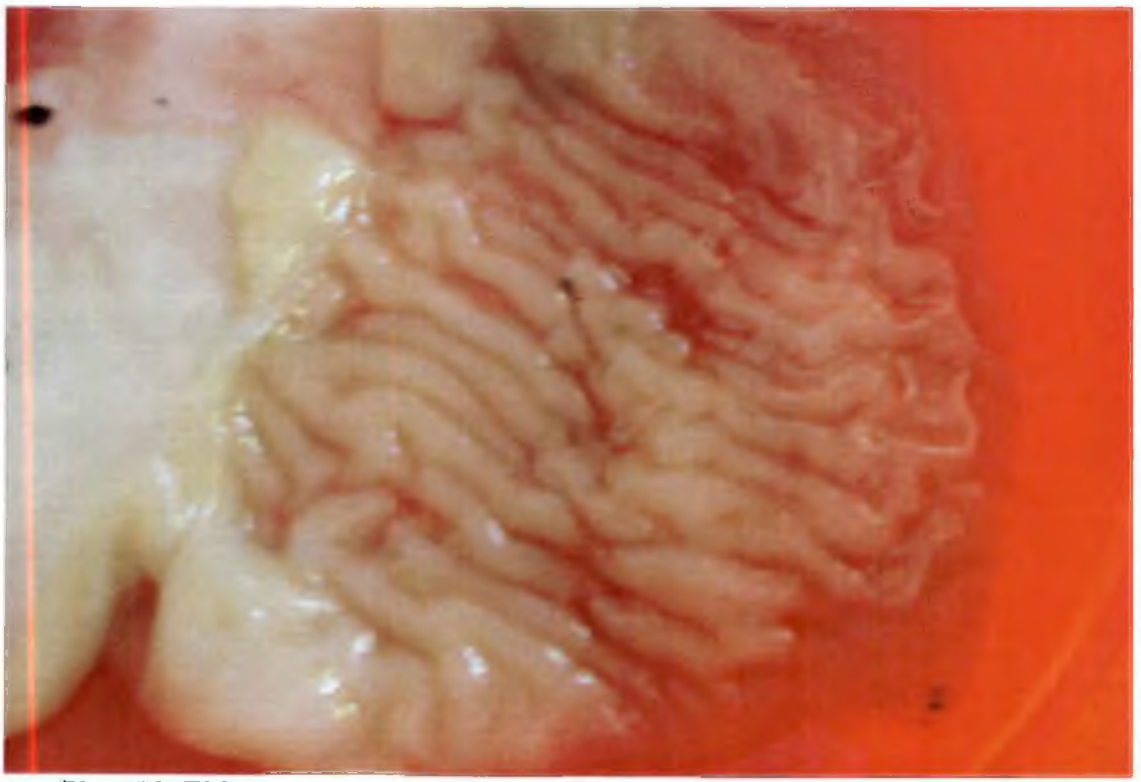

Plate 13. Thickened corrugated ileum from a goat with paratuberculosis

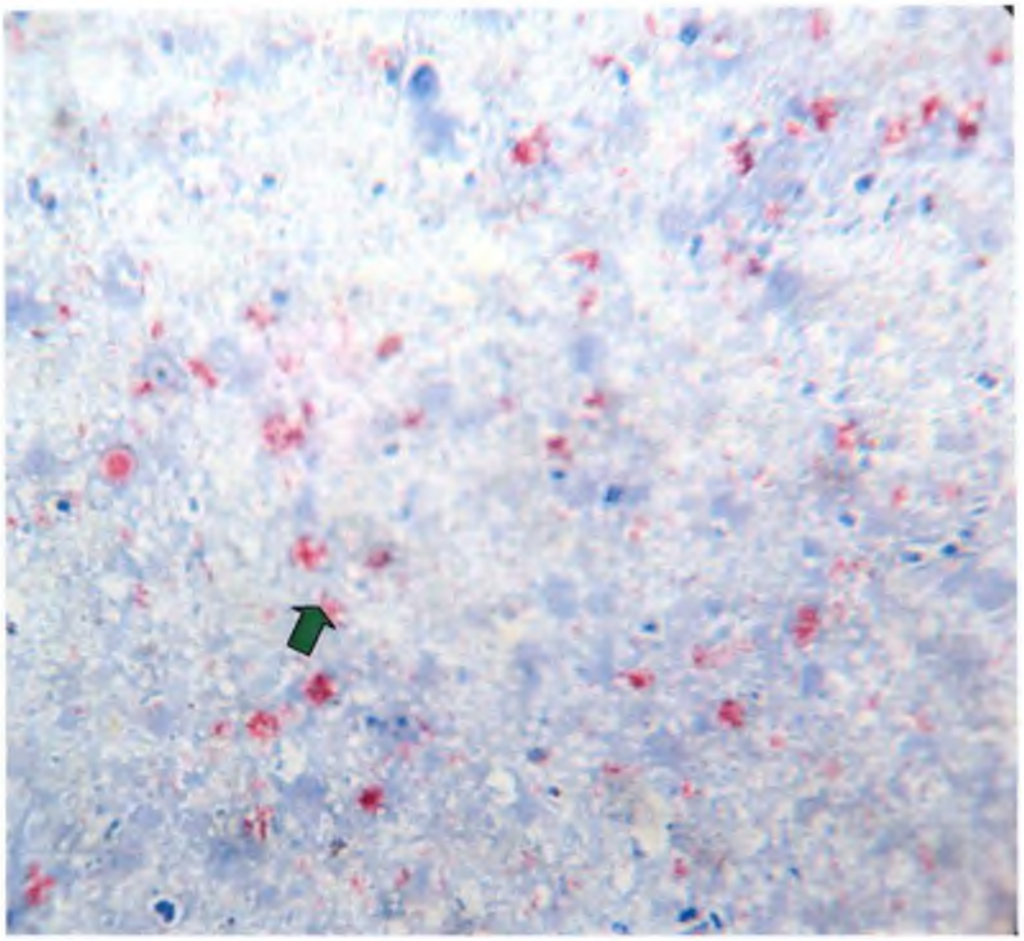

Plate 14. Acid fast bacilli in mesenteric lymph node impression smear

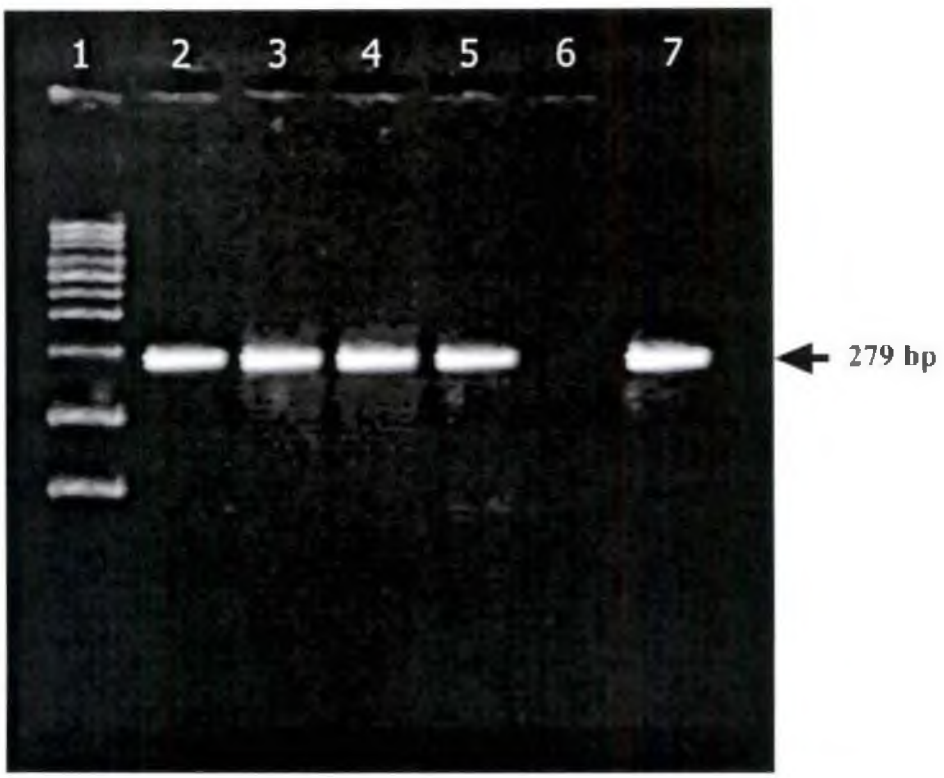

Plate 15. PCR for the amplification of IS*900* gene of MAP

Lane 1 100 bp DNA Ladder

Lane 2 to 5 Samples positive by PCR

Lane 6 Negative control

Lane 7 Positive control (279 bp)

4.2 COMPARISON OF EFFICACY OF ZIEHL-NEELSEN ACID FAST STAINING, SINGLE INTRADERMAL JOHNIN TEST AND PCR IS*900*

The comparative efficacy for detecting paratuberculosis positive goats of USGF, Mannuthy by above three tests are presented in Table (4) and that from field is given in Table (5). Statistical analyses of the results obtained were done by chi square and proportion test (Rangaswamy, 1995). The result of PCR was significant at one per cent level when compared with acid fast staining and single intradermal test.

4.2.1 Comparison of acid fast staining and single intradermal Johnin test

Comparison of acid fast staining and single intradermal Johnin test is presented (Figures 1, 2 and 3). Proportion (Z) test for acid fast staining and single intradermal Johnin test did not yield any significant difference.

4.2.2 Comparison of acid fast staining and IS*900* PCR

Comparative efficacy of these tests is presented in Figures (7, 8 and 9). The efficacy of polymerase chain reaction was significantly high at one per cent level when compared with acid fast staining in detecting paratuberculous goats and comparative efficacy of PCR was more.

4.2.3 Comparison of single intradermal Johnin test and IS*900* PCR

Efficacy of these two tests in detecting paratuberculosis positive goats was compared and it is given in Figures (4, 5 and 6). Polymerase chain reaction was more efficient in detecting Johne's disease in goats and it was significantly different (at one per cent level) from single intradermal test.

4.2.4 Comparison of acid fast staining, single intradermal Johnin test and IS*900* PCR

Comparative efficacy of these three tests in diagnosing paratuberculosis in goats is presented in Figures (10, 11, 12, 13, 14, 15 and 16). The combined comparative test results from goats of USGF, Mannuthy and that from field is given in Table (6). Among the three tests under study for diagnosis of paratuberculosis in goats, it was found that PCR was significantly different (at one per cent level) from Ziehl-Neelsen acid fast staining and single intradermal Johnin test . Chi square (x^2) value for the three tests was significant at one percent level for both the group of animals .

Results of test are uniform in goats of USGF, Mannuthy and goats from field. There was no significant difference for Ziehl-Neelsen acid fast staining and single intradermal Johnin test. But IS*900* PCR was significantly different from other two tests indicating that IS*900* PCR was more efficient test for diagnosing paratuberculosis in goats.

Table 1. Faecal smear examination by Ziehl-Neelsen acid fast staining

	No of animals tested	No of goats positive for MAP	Positive%
USGF MANNUTHY	150	5	3.33
FIELD	50	1	2
TOTAL	200	6	3

Table 2. Single intrademal Johnin test

	No of animals tested	No of goats positive for MAP	Positive%
USGF MANNUTHY	150	8	5.3
FIELD	50	3	6
TOTAL	200	11	5.5

Table 3. Polymerase Chain Reaction IS*900*

	No of animals tested	No of goats positive for MAP	Positive%
USGF MANNUTHY	150	30	20
FIELD	50	12	24
TOTAL	200	42	21

Table 4. Comparative results of three tests for USGF,Mannuthy

Test	No of animals tested	No of goats positive for MAP	Positive%
Acid fast staining	150	5 NS	3.33
Johnin SID	150	8 NS	5.3
PCR IS*900*	150	30 **	20

**Chi-square (x^2) value for farm is 28.74 (significant) P< 0.01.

Table 5. Comparative results of three tests for field animals

Test	No of animals tested	No of goats positive for MAP	Positive%
Acid fast staining	50	1 NS	2
Johnin SID	50	3 NS	6
PCR IS*900*	50	12 **	24

**Chi-square (x^2) value for field is 14.14 (significant) P< 0.01.

Table 6. Combined comparative test results for farm and field

Test	No of animals tested	No of goats positive for MAP	Positive%
Acid fast staining	200	6 NS	3
Johnin SID	200	11 NS	5.5
PCR IS*900*	200	42 **	21

**Chi-square (x^2) = 42.89608 is significant. Table value at 1 percent level is 9.21. P< 0.01

Table 7. Post mortem findings in single intradermal (SID) Johnin test positive animals

Sl No	Goat No	SID difference in skin thickness	Period between SID and slaughter	Gross lesions in			Light microscopy of mesenteric lymph node impression smear.
				Intestine	Mesenteric lymph node	Other organs	
1	1390	6mm	267 days	No gross lesions	No gross lesions	No gross lesions	Acid fast bacteria present
2	1706	4mm	237 days	Corrugated ileum	No gross lesions	Gelatinization of fat, atrophy of skeletal muscle	Acid fast bacteria present
3	880	10mm	161 days	No gross lesions	Enlarged & edematous	No gross lesions	Acid fast bacteria could not be detected

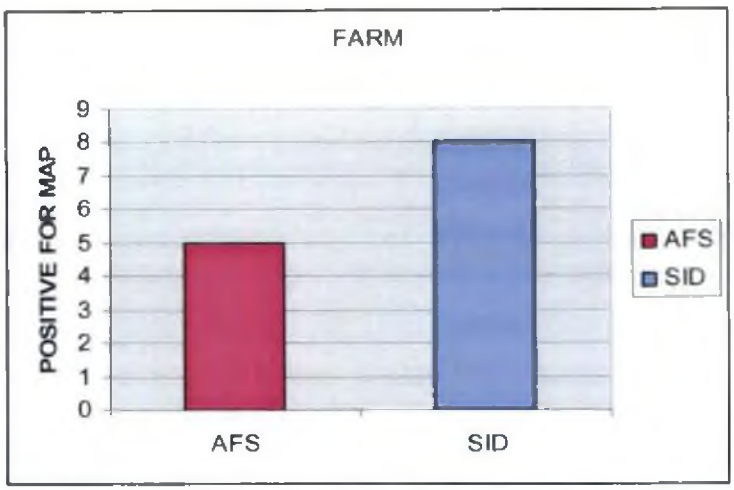

Fig.1.Comparison of acid fast staining and single intradermal test

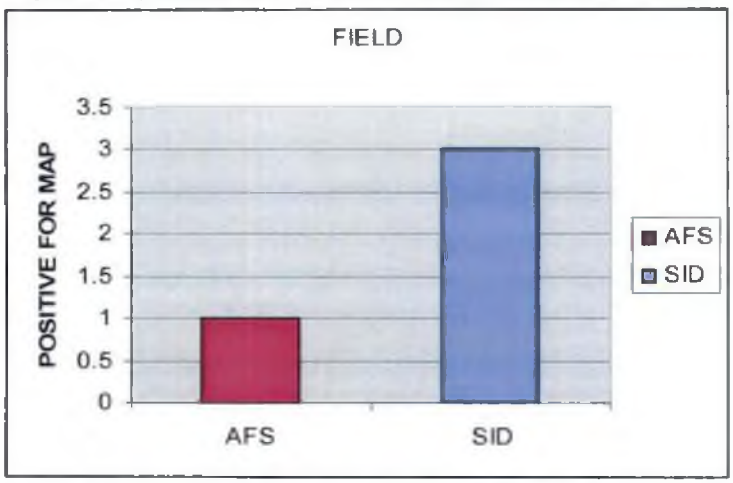

Fig.2.Comparison of acid fast staining and single intradermal test

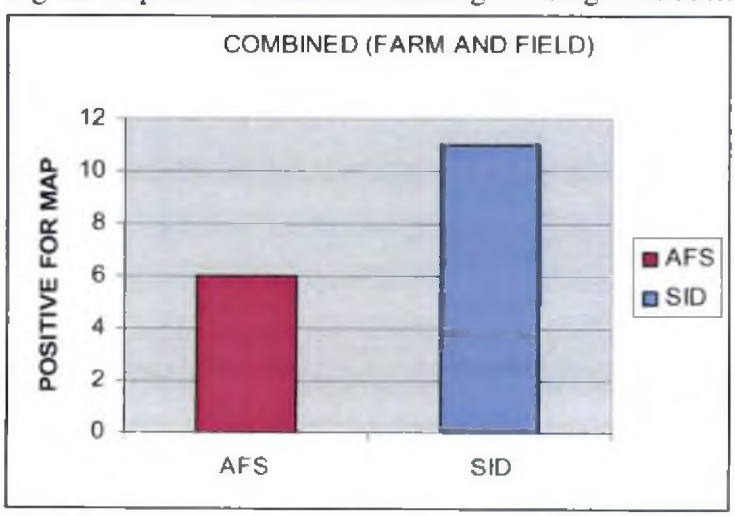

Fig.3.Comparison of acid fast staining and single intradermal test

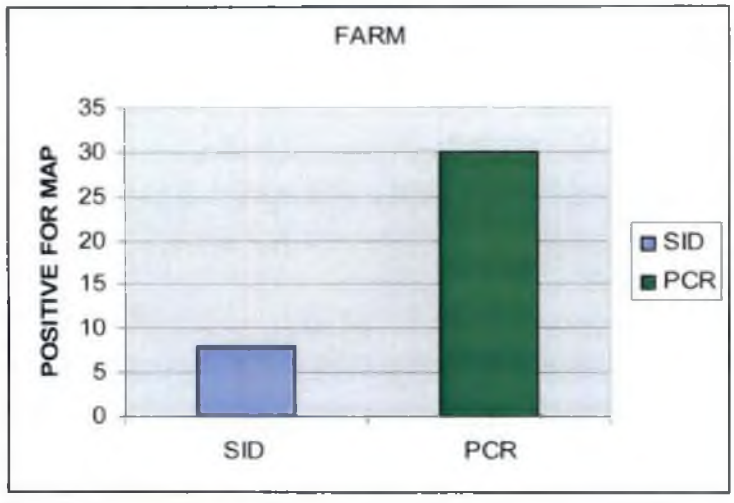

Fig.4. Comparison of single intradermal test and PCR

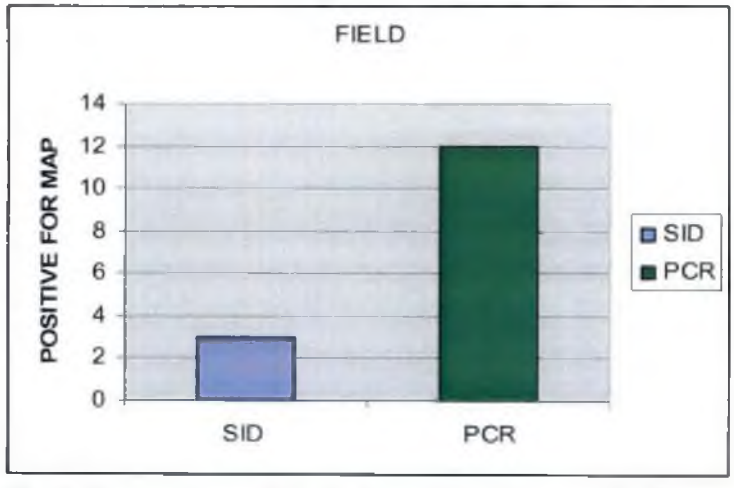

Fig.5. Comparison of single intradermal test and PCR

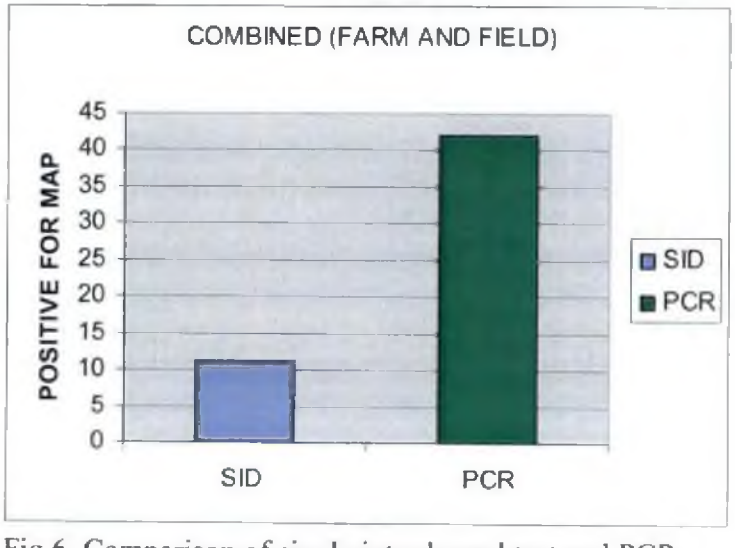

Fig.6. Comparison of single intradermal test and PCR

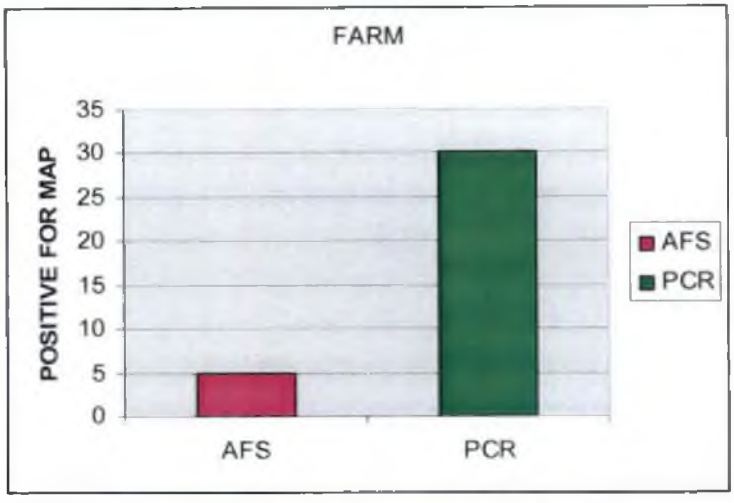

Fig.7. Comparison of acid fast staining and PCR

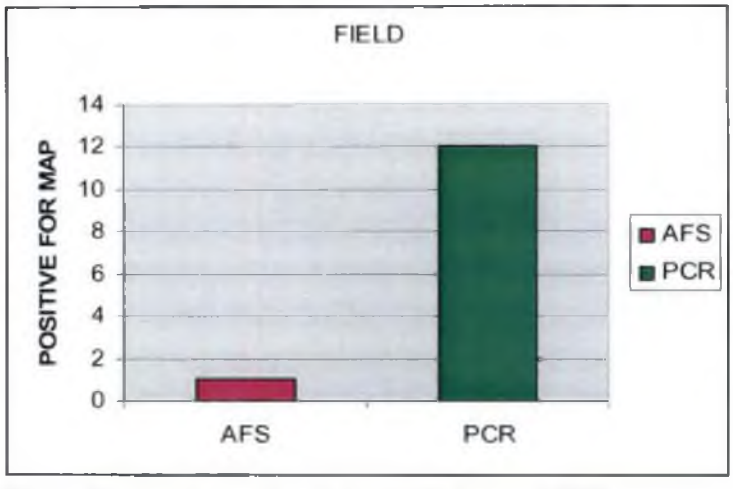

Fig.8. Comparison of acid fast staining and PCR

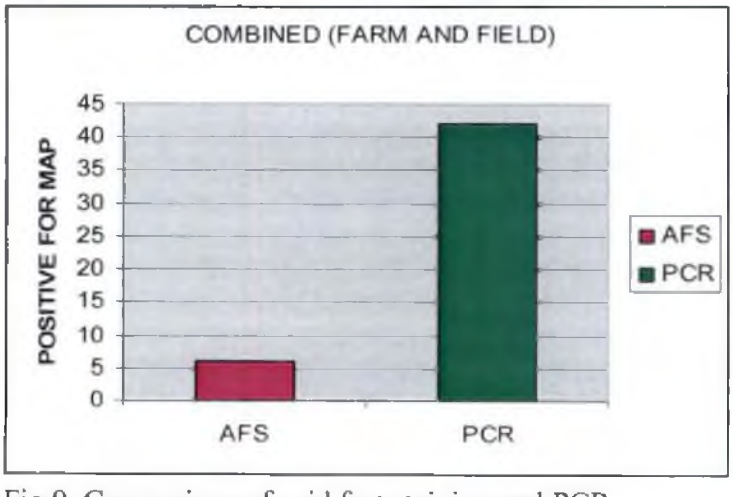

Fig.9. Comparison of acid fast staining and PCR

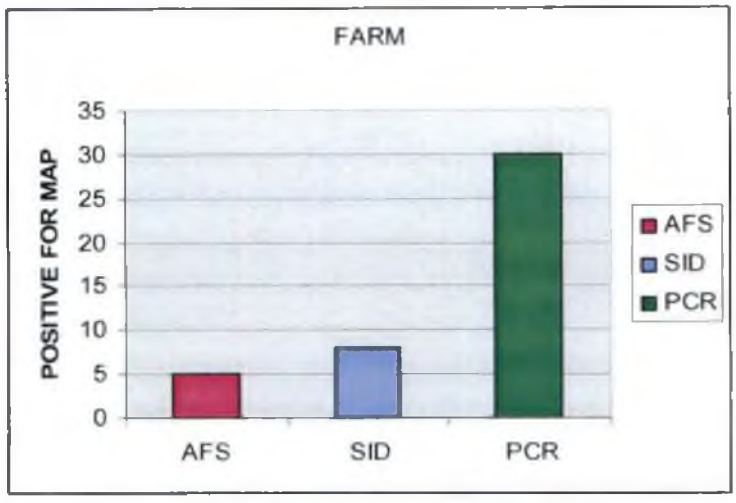

Fig.10. Comparison of acid fast staining, single intradermal test and PCR

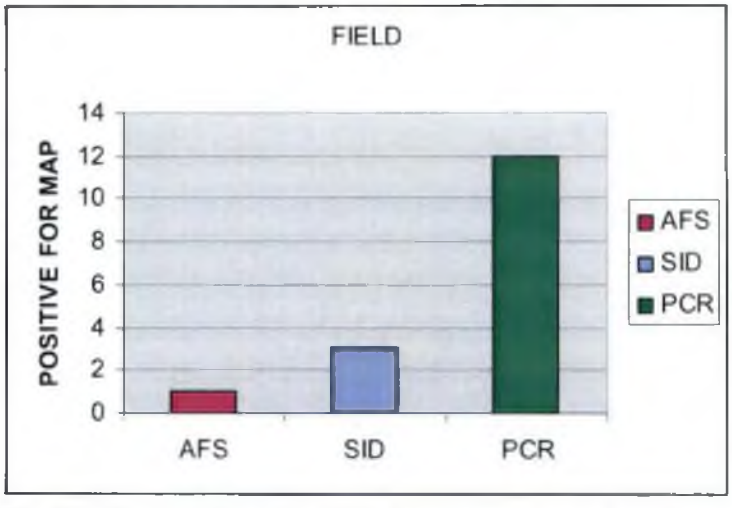

Fig.11. Comparison of acid fast staining, single intradermal test and PCR

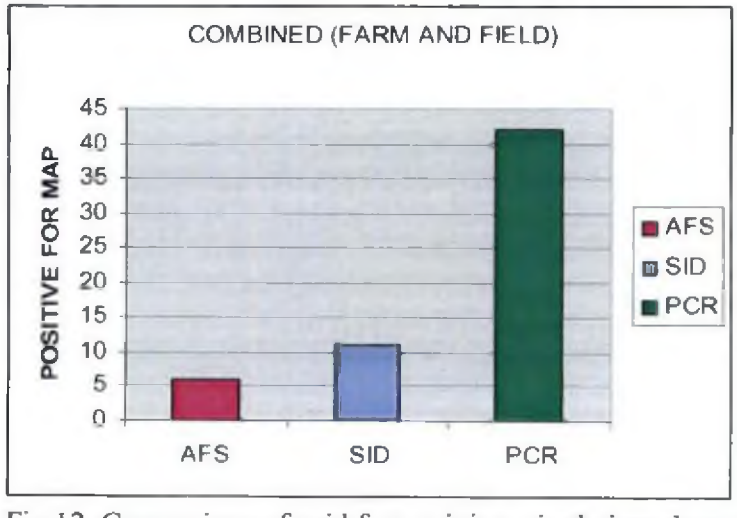

Fig.12. Comparison of acid fast staining, single intradermal test and PCR

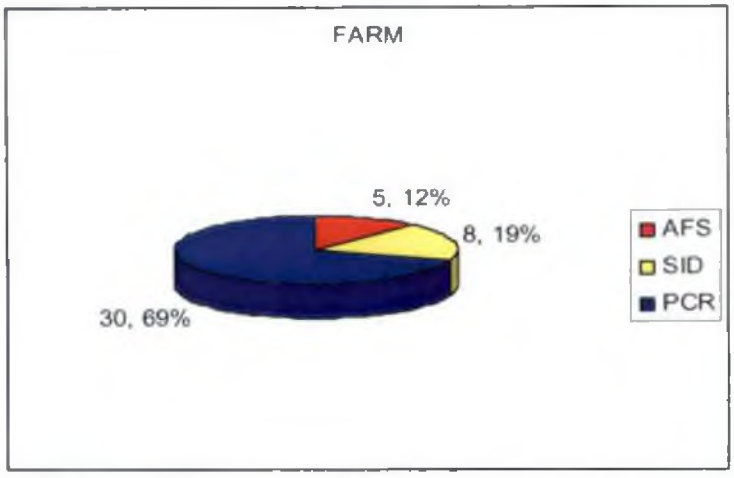

Fig.13. Comparison of acid fast staining, SID Johnin and PCR

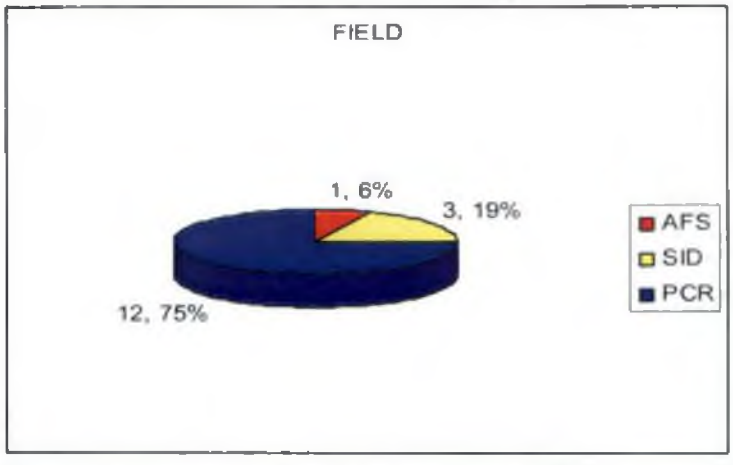

Fig.14. Comparison of acid fast staining, SID Johnin and PCR

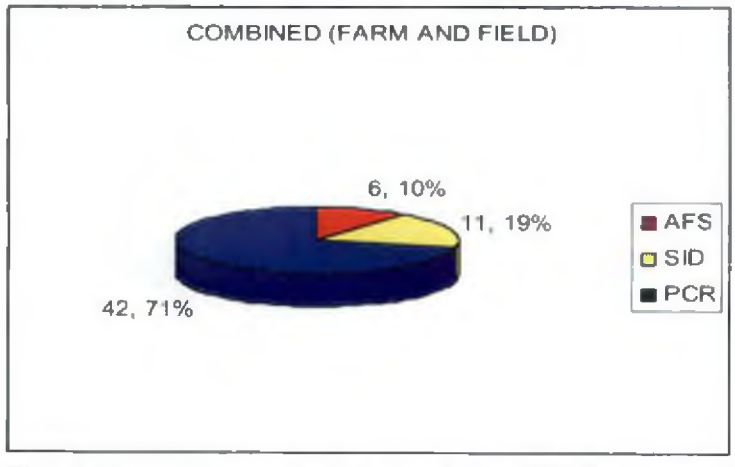

Fig.15. Comparison of acid fast staining, SID Johnin and PCR

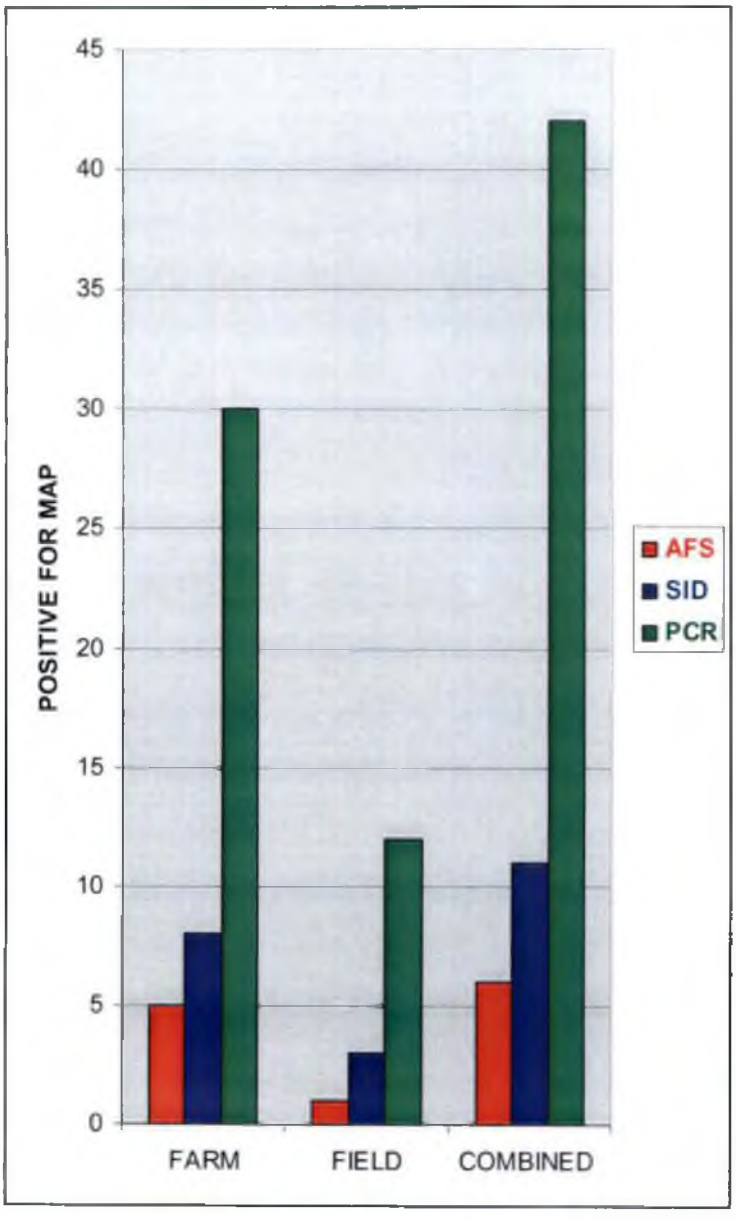

Fig.16. Comparison of acid fast staining (AFS), single intradermal Johnin (SID) and PCR. (150 animals from farm, 50 animals from field and combined result for 200 animals)

Discussion

5.DISCUSSION

Though paratuberculosis was described more than a century ago and the causative agent identified, yet the disease continues to frustrate the scientific and agricultural communities. Chronicity of the disease development is a hallmark of paratuberculosis and extensive efforts have been directed towards an effective means of diagnosing the non clinical carrier animals which are capable of spreading *Mycobacterium avium* subsp *paratuberculosis* to numerous herd mates. Successful control and eradication of paratuberculosis has been largely impeded by the lack of accurate diagnostic tests that are able to correctly classify individuals, especially at the early stages of infection. A great deal of progress has been made in the development of early diagnostic techniques and control measures for minimizing spread and elimination of disease on a herd basis. Although a number of diagnostic tests are available for Johne's disease, debate exists over which is the most effective. Paratuberculosis has not been studied in caprine species so well, as in cattle and sheep. Therefore, the present study was carried out to record the most suitable diagnostic test for early and accurate diagnosis of this disease in goats.

5.1 COMPARATIVE EFFICACY OF VARIOUS DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS

In the present study, direct microscopic examination of faecal smear by Ziehl-Neelsen acid fast staining, single intradermal Johnin test and Polymerase chain reaction IS*900* diagnostic test for detection of *Mycobacterium avium* subsp *paratuberculosis* (MAP) were evaluated to identify the most effective test for diagnosis of paratuberculosis in two hundred goats and the results were presented in Table (1 to 6) and Fig. (1 to 16).

5.1.1 Microscopic faecal smear examination by Ziehl-Neelsen's acid fast staining

5.1.1.1 University sheep and goat farm (USGF) Mannuthy

Out of one hundred and fifty animals screened for paratuberculosis by acid fast staining of faecal smear, five were found to be positive for MAP (Table 1 and 4 and Figures 1, 7, 13, and 16). The faecal samples from all the five animals showed clumps of acid fast bacilli (Plate 2). Direct microscopy of acid fast stained faecal smear examination was considered as significant diagnostic test in herd screening of paratuberculosis in goats (Hajra *et al.*, 2007). But the present finding is not in agreement with this observation since the percentage of detectability of paratuberculosis in goats by this test was only 3.33 per cent (Table 1 and 4). Hole (1952) found that excretion of bacteria in pre clinical stage would be very poor. Hence positive result for Ziehl-Neelsen acid fast staining depends on stage of disease at the time of collection of sample as this test is having very low sensitivity and specificity (Moser, 1982 ; Zimmer *et al.*, 1999). Doyle and Spears (1951) recorded that examination of faecal smears from apparently normal animals in infected herd with the objective of detecting early case of the disease appeared to be of little value. This could be one of the reasons for getting the poor detection rate in the present study with acid fast staining, as most of them might be in an early stage of infection, though in an infected herd. Since the animals in the University sheep and goat farm, Mannuthy were periodically subjected to intradermal Johnin skin test and the positive animals were culled, the chance of finding more number of animals in the advanced stage of infection was also very little.

5.1.1.2 Test result of field

Out of fifty animals screened for paratuberculosis from field by acid fast staining only one animal was found positive (2 per cent) (Table 2 and 5, Figures 2, 8, 14 and 16). Microscopical examination of faecal sample has a marked positive value in clinical disease, but a negative result has no significance. Direct microscopy of faecal smear by acid fast staining is a low specificity test (OIE,

2004). Doyle (1956) considered that only 25 to 30 per cent of cases were identifiable by this means at the first examination. The chief advantage of this method is its positive accuracy, as the presence of one typical clump is accepted as indisputable evidence. Allergic test was less reliable both in preclinical and in very advanced stage of infection and faecal sample examination was helpful in detecting animal shedding the organism in advanced clinical stage (Paliwal *et al.*, 1984). But present finding was in contradiction to above observation which could be explained by the finding of Singh *et al.* (2007a), who reported that performance of particular diagnostic test depended on stage of disease. Delayed type hypersensitivity tests are usually positive before faecal culture and serology or clinical signs. Thus detection of paratuberculosis in a herd at a single point of time with a single diagnostic test is difficult.

In the present study, acid fast staining of faecal smear has given lowest sensitivity for paratuberculosis in goats both at farm and field levels. It is impossible to diagnose all paratuberculous goats by faecal smear examination because of the slow progression of the disease, intermittent shedding of MAP in faeces and often low level shedders are also missed. Acid fast staining has limited sensitivity and as many as 10^6 bacteria per gram is necessary for detection of acid fast rods (Thoresen *et al.*, 1994). With light microscopy only heavy shedders in advanced stages of clinical cases could be detected, that too with 25-30 per cent cases identifiable by first examinations of faecal sample. Result of the present study, that is reduced detection rate of MAP in faeces by light microscopy, is in agreement with the findings of Moser (1982), Chiodini *et al.* (1984) and Thoreson *et al.* (1994).

5.1.2 Single intradermal Johnin test

5.1.2.1 University sheep and goat farm (USGF) Mannuthy

Among one hundred and fifty animals tested by single intradermal Johnin test eight animals were found positive for MAP (Table 2 and 4 and Figures 1, 4, 10, 13, and 16). The results indicated that 5.3 per cent of tested animals were in the initial stages of infection, giving a positive cell mediated immune response. Positive animals were eliminated from the herd every year and hence this could be the reason for low positivity in the herd. Reactions to allergic test are more marked in the initial stages of infection and becomes less marked as the disease become established (Hole and Maclay 1959). Cell mediated immune response is considered to be the first and the most important response of animals to infection with mycobacteria (Collins, 2003b). Johnin skin test and its *in vitro* equivalent IFN-γ assay are tests measuring cell mediated immunity that help to control paratuberculosis by diagnosing mycobacterial infection earlier, before animal become infectious. Results of the post mortem findings of single intradermal Johnin test positive animals from farm (Table 7) denoted that in one animal there was no visible lesion 267 days after single intradermal test and the test was able to identify the infected animal at an early stage of infection . Postmortem confirmation of the disease was made possible only by demonstrating acid fast bacteria in lymph node impression smear. The animals that were slaughtered 237 days and 161days after single intrademal test had shown visible lesions in ileum and lymph node respectively.

5.1.2.2 Test result of field

When fifty animals were screened for paratuberculosis from field three were found positive (6 per cent) by single intradermal Johnin test (Table 2 and 5 and Figures 2, 5, 11, 14 and 16). Present finding is not in agreement with that of Paliwal *et al.* (1984) who opined that acid fast staining of faecal smear was better than Johnin single intradermal test in diagnosing paratuberculosis in sheep. But

NRC (2003) suggested that for each and every stage of disease the result given by a particular diagnostic test also varied. Here cell mediated immune response is measured which gives more positive results in the early stage of the infection before faecal shedding of organism begins.

Compared to acid fast staining , single intradermal Johnin test has given a positive test result of 5.5 per cent which is 2.5 per cent more (Fig. 3). Single intradermal test has high sensitivity in goats with paucibacillary lesion (Tripathi *et al.,* 2006). Skin test using intradermal Johnin measuring delayed type of hypersensitivity is said to be the best test to identify positive animals before clinical disease develops and is widely used in field condition (Julian, 1975). The high rate of positivity in single intradermal Johnin test than acid fast staining of faecal smear examination indicated that more goats were in the early stages of infection when the screening was done. The results of intradermal injection of Johnin might give false negative results in very early and late stage of infection, advanced stage of pregnancy and just after parturition and within 60 days of Johnin test or false positive reactions in conditions like animals sensitized to other allergen, infected with non pathogenic saprophytic mycobacteria and vaccination (Lalkrishna, 2007).

Animal in advanced stage of pregnancy or just after parturition or within 60 days of single intradermal Johnin test were not utilized for conducting Johnin test and hence false negative due to this reason did not arise. Since vaccination against paratuberculosis is practised no where in Kerala chances of getting false positive results on that account is also not possible. Hence the results obtained by single intradermal Johnin test can be considered true positive and such animals will be in the stage two of the disease, which is indicated by cell mediated immune response. The single intradermal test result in present study showed that Johnin test was more efficient in detecting MAP infection than acid fast staining, since the early cell mediated immune response was detected by delayed type hypersensitivity using Johnin in animals which were infected after last culling. In a study, skin test had been found to be as sensitive as IFN-γ assay in ovine paratuberculosis (Perez *et al.,* 1999). The infection with MAP presents a spectrum of immune responses which

may vary from the initial appearance of cell mediated immunity followed by humoral response to ultimately terminating into anergy (Chiodini *et al.*, 1984). Hence cell mediated immune response based assays are practically useful for the early detection of infection.

5.1.3 Results of post mortem examination

Post mortem examination of slaughtered animals revealed that all the single intradermal test positive animals had paratuberculosis infection. From mesenteric lymph node impression smear, acid fast organism could be detected in two animals and one animal had shown typical lesion suggestive of Johne's disease (enlarged oedematous lymph node). One animal showing acid fast bacteria in mesenteric lymph node had also shown corrugations in ileum and in that goat gelatinization of fat depot and atrophy and dehydration of skeletal musculature could also be observed.

Basically the lesions of paratuberculosis remained confined to intestine and regional lymph nodes (Paliwal and Rajya, 1982). The result of present study is in agreement with this observation. National research council (2003) stated that the most common gross lesions were thickening of terminal small intestine, enlargement of mesenteric lymph node and corrugation of the ileal mucosa. Sharma *et al.* (1998) reported that the lesions were more evident in the jejunum, ileum, caecum and associated mesenteric lymph node in most of the cases. Acid fast bacteria appeared free in the cortical and paracortical areas in the lymph node. Corrugation of ileum, swollen and enlarged lymph node and presence of acid fast bacteria in mesenteric lymph node impression smear were also observed in this study. In one animal typical changes in lymph node were observed but the organism could not be detected from impression smear of lymph node. It could be due to the fact that in paucibacillary form acid fast bacteria were rarely detected from mesenteric lymph node. In the animals with paucibacillary lesion had well developed Th 1 immiune response and uncontrolled multiplication of MAP was prevented (Reddacliff, 2002).

5.1.4 Polymerase Chain Reaction IS*900*

5.1.4.1 University sheep and goat farm (USGF) Mannuthy

Out of hundred and fifty animals tested by PCR for MAP, thirty animals were found positive (Table 3 and 4 and Fig 4, 7, 10, 13 and 16). Among all these tests, PCR detected maximum number of infected animals (20 per cent). This finding is in agreement with Socket *et al.* (1992) who reported that IS*900* PCR testing of faeces in cows was highly specific and sensitive, when faecal shedding was frequent. Polymerase chain reaction had been used to identify samples that were culture negative as well as detected fento gram (less than two genome copies) amount of DNA (Huntley *et al.*, 2005). Shivakumar *et al.* (2005) reported that PCR was more sensitive than smear examination in diagnosis of Johne's disease. The apparent specificity of IS*900* for MAP was quickly exploited by using PCR to develop a rapid assay for identification of MAP from clinical specimens (Vary, 1990).

5.1.4.2 Test result of field

Out of fifty animals twelve were found positive by PCR (24 per cent) (Table 3 and 5 and Figures 5, 8, 11, 14 and 16). Moss *et al.* (1991) reported that with PCR only a small amount of DNA was required and purity of the sample was not always critical. Narang and Kaur (2007) opined that, DNA probe could be used to detect presence of DNA in faeces and PCR was able to detect as low as 50 organism per gram of faeces. This could be the reason for the maximum number of positive results obtained from field by PCR. Whitaker (2005) found that PCR could be used to eliminate subclinical shedders from herd and thereby reduce environmental contamination. At present, all tests have limitation at certain points during progression of MAP infection. From the findings of present study it is clear that PCR could be used for the early and accurate diagnosis of paratuberculosis at any stage of the disease.

5.2 COMPARISON OF EFFICACY OF ZIEHL-NEELSEN ACID FAST STAINING, SINGLE INTRADERMAL JOHNIN TEST AND PCR IS*900*

The comparative efficacy of these three tests in diagnosing paratuberculosis in goats was presented (Table 6 and Figures 12, 15 and 16).

5.2.1 Comparison of acid fast staining and single intradermal Johnin test

Comparison of acid fast staining and single intradermal Johnin test is presented (Table 4, 5 and 6 and Figures 1, 2 and 3). In farm out of one hundred and fifty animals screened by acid fast staining of faecal smear, five animals were found positive (3.33 per cent). Single intradermal Johnin test could detect eight positive animals (5.3 per cent). Though there wasn't any statistically significant difference between the two tests, single intradermal Johnin test was superior to acid fast staining of faecal smear in diagnosing positive animals from the farm. Annual screening by single intadermal Johnin test and culling of the goats at University sheep and goat farm, Mannuthy could be one important factor for the low number of advanced cases in farm.

When comparative efficacy of these two tests were evaluated at field level, out of fifty goats screened one animal (2 per cent) was positive by acid fast staining and three animals were found positive (6 per cent) by single intradermal Johnin test (Fig 2). For field also Johnin test gave a better result compared to acid fast staining though there wasn't any statistically significant difference between the two tests under comparison. This finding also points that farmers are selling out weak and debilitated goats, rather than keeping and treating. Hence there is less chance for much faecal shedders.

Combined test results of farm and field cases revealed that six animals were positive (3 per cent) for acid fast staining of faecal smear and eleven animals (5.5 per cent) positive for single intradermal Johnin test (Fig 3). Proportion test for acid fast staining and single intradermal Johnin test did not yield any significant difference. But more positive results for single intradermal Johnin test indicated that the infection was persisting in the goat population. Infected animals are detected during early stages of infection by tests like single intradermal Johnin test and PCR.

Results of the present study agree with findings of Kandavel and Nedunchelliyan (1987), who got better results for detection of paratuberculosis in cattle with single intradermal Johnin test than that with microscopy of Ziehl-Neelsen stained faecal smear.

5.2.2 Comparison of acid fast staining and IS*900* PCR

Comparative efficacy of Ziehl-Neelsen acid fast staining and IS*900* PCR for diagnosis of paratuberculosis were presented (Figures 7, 8 and 9). Acid fast staining of faecal smear from farm detected five animals out of one hundred and fifty (3.33 per cent) while PCR had given positive results for thirty animals (20 per cent) (Table 4 and Fig 7)). There was significant difference in the diagnostic capability of the two tests under comparison, with maximum number of infected animals being detected by PCR.

A similar result was obtained from field animals; out of fifty animals screened for paratuberculosis by acid fast staining one animal (2 per cent) and by IS*900* PCR twelve animals (24 per cent) were found positive for paratuberculosis. Significant statistical difference was observed between these two tests (Table 5 and Fig 8).

Combined comparative results from farm and field revealed that six (3 per cent) and forty two animals (21 per cent) were positive for paratuberculosis by acid fast staining and IS*900* PCR respectively (Table 6 and Fig 9).The efficacy of PCR was significantly high when compared with acid fast staining in diagnosing

paratuberculous goats. The high sensitivity of PCR and annual culling of positive animals could be the contributing factors for the significant difference of PCR from that of acid fast staining in case of farm animals.

Only 25 to 30 per cent of cases were identifiable by first examination of faecal sample (Doyle, 1956). Hole and Maclay (1959) stated that microscopical examination of faecal sample had a marked positive value in clinical cases, but a negative result had no significance. Since faecal shedding is not a consistent feature of subclinical infection, faecal smear examination is less reliable because greater number of low level shedders might be missed giving large number of false negative results (Moser, 1982). Thoresen *et al.* (1994) opined that acid fast staining had a limited sensitivity and as many as 10^6 bacteria per gram was necessary for detection of acid fast rods by light microscopy. Probe test detected MAP DNA in faecal specimen from animals shedding at least 10^4 MAP colony forming unit per gram of faeces (Whipple *et al.,* 1992). The animals screened in this experiment might not be in the advanced clinical stage of the disease, so only six animals are detected by acid fast staining of faecal smear. Possibility for forty two animals being diagnosed by PCR could be due to its ability to detect even $1/100^{th}$ number of bacteria in faecal sample when compared to faecal smear examination by light microscopy.

5.2.3 Comparison of single intradermal Johnin test and IS900 PCR

Efficacy of these two tests in detecting paratuberculosis positive goats was compared and it is given (Figures 4, 5 and 6). From farm out of one hundred and fifty animals screened by single intradermal Johnin test, eight animals (5.3 per cent) and by IS*900* PCR thirty animals (20 per cent) were found positive for MAP (Table 4 and Fig 4). The efficacy of polymerase chain reaction was found to be significantly higher when compared with single intradermal Johnin test.

Results obtained from the field animals also agreed with the above findings. Out of fifty goats screened, three were found to be positive by single intradermal (6

per cent) and twelve animals (24 per cent) by PCR (Table 5 and Fig 5) indicating a significant statistical difference between the tests under comparison.

Combined comparative efficacy of these two tests for animals from both farm and field was presented (Table 6 and Fig 6). Combined results followed a pattern that was similar to the individual results of farm and field. Out of two hundred animals screened eleven animals (5.5 per cent) and forty two animals (21 percent) were positive for single intradermal Johnin test and IS*900* PCR respectively (Fig 6). Proportion test yielded a significant difference for PCR indicating its increased efficiency in diagnosis of paratuberculosis, compared to single intradermal test. Moss *et al.* (1991) reported that for PCR only a small amount of DNA was required and purity of the sample was not always critical. Polymerase chain reaction had been used positively to identify samples that were culture negative and detected less than two genome copies of DNA (Huntley *et al.*, 2005). Narang and Kaur (2007) reported that, DNA probe could be used to detect the presence of DNA in faeces and PCR was able to detect as low as 50 organism per gram of faeces. Hence chances of detecting fento gram level of DNA in various stages of the disease might have resulted in a high positivity for PCR, comparing single intradermal Johnin test and faecal smear examination.

5.2.4 Comparison of acid fast staining, single intradermal Johnin test and IS*900* PCR

Comparative efficacy of these three tests in diagnosing paratuberculosis in goats is presented in Figures (12, 15 and 16). The combined comparative test results from goats of University sheep and goat farm, Mannuthy and that from field is given in (Table 6).

While comparing the results of acid fast staining of faecal smear and single intradermal Johnin test for the diagnosis of paratuberculosis in goats there wasn't any significant difference in their detectability in Johne's disease positive animals. But when PCR was compared with acid fast staining and intradermal Johnin test,

PCR was found to be significantly different from other two tests (Table 6 and Figures 12, 15 and 16).

Out of two hundred animals screened for paratuberculosis 42, 11 and 6 animals were found positive by PCR, single intradermal Johnin test and acid fast staining of faecal smear respectively. When a combination of tests were considered at a time *ie*, single intradermal and PCR, acid fast staining and PCR and single intradermal and acid fast staining, and the number of animals falling positive to paratuberculosis in each category was analysed, it was observed that for single intradermal and PCR only two animals were found positive. Possible reason for two animals being positive to these tests was that the animals might be in stage II of the disease. Perez *et al.* (1999) in a study in naturally acquired paratuberculosis in sheep found that IFN-γ assay was almost equal to skin test in diagnosing MAP infection. National research council (2003) stated that in stage II of the disease PCR might give a positive result and IFN-γ assay would give a sure positive result. Animals in stage II or subclinical stage of the disease had altered cellullar immune response and intermittently shed low level of organism. Here the altered cellular immune response was measured by single intradermal Johnin test and low level shedding of organism was detected by PCR.

When acid fast staining and PCR were taken at a time and the results analysed, four animals were found positive for paratuberculosis by these tests. Polymerase chain reaction could detect fentograms of DNA (Huntley *et al.*,2005). For detection of acid fast bacteria in smear examination by Ziehl-Neelsen staining it needs 10^6 bacteria per gram (Thoresen *et al.,* 1994). So naturally the cases found positive by acid fast stained faecal smear have to be positive in PCR also, since the detection limit of PCR according to Nandi (2008) was 50 organism per gram of faeces. This is a valid reason for the result obtained in present study.

All the six positive cases detected by acid fast staining were found positive for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by PCR IS*900* also, indicating that the organism identified was not any non pathogenic saprophytic mycobacteria and the fact that the product amplicon size was 279 bp suggested that the organism identified in the faecal smear was MAP. Polymerase chain reaction

was capable of detecting 36 more samples than that by acid fast staining since PCR needs only 10^4 organism per gram of faeces for detection (Whipple *et al.*, 1992). Acid fast staining of faecal smear is positive only when the organism is frequently discarded in faeces and for detection by faecal smear it needs about 10^6 organisms per gram of faeces. Intermittent shedders and low level shedders by direct microscopy of acid fast stained faecal smear often gave false negative results (Moser, 1982).

While considering the results of single intradermal test and acid fast staining, none among the two hundred goats gave a positive result for these two tests at a single point of time. As a general rule the result has to be like that. Skin test is quite sensitive soon after infection (Gilmour *et al.*, 1978). Hole and Maclay (1959) reported that as the disease became established the allergic test became less marked. Moser (1982) observed that intradermal test was unreliable due to high false negative result in late stage of the disease. Johnin test was helpful in identifying early stage of the disease but in later clinical stage animal might not evoke enough response (Roy *et al.*, 2004). Johnin test had the potential to detect animals infected with MAP early in the course of disease (Austerman *et al.*, 2007). Julian (1975) opined that faecal smear examination for acid fast organism was useful to pick out shedders in a known positive herd. To get positive results in Ziehl-Neelsen stained faecal smear the animal should have severe lesion and should be in advanced stage of the disease (Whittington *et al.*, 2000). Cell mediated immune response test is used for diagnosing infection in earlier stage (stage I or II) where as acid fast bacilli are seen in faecal smear in stage III or IV of the disease (NRC, 2003). Perez *et al.* (1999) observed that most sheep with scant or no acid fast bacilli in the intestine had positive results in IFN-γ assay and skin test whereas most sheep with diffuse lesion and high number of bacilli (multibacillary) were test negative. Animals with clinical paratuberculosis may exhibit low CMI and thus present negative IFN-γ test results at stages of the disease at which they are positive by other tests (Jungersen *et al.*, 2002). Hence the chances are very limited for getting positive results in common for tests that are used to measure the two extremes of the disease and these results are agreement with findings of previous

workers (Hole and Maclay 1959; Julian, 1975; Gilmour *et al.,* 1978; Moser, 1982; Whittington *et al.,* 2000; NRC, 2003; Roy *et al.,* 2004; Austerman *et al.,* 2007).

Only two animals were found to be positive for acid fast staining of faecal smear, single intradermal Johnin test and PCR. Most of the references cited just above argued that when acid fast organism is excreted at a level detectable by faecal smear examination the cell mediated immune response has to be waned. But here instead two goats diagnosed as paratuberculous positive by acid fast staining and PCR showed CMI response also. Sharma *et al.* (1985) identified acid fast bacilli from faeces of five sheep out of fifty eight sheep tested positive for single intradermal Johnin test. Single intradermal Johnin test was positive in 58.1 per cent of faecal smear positive cows (Kormendy, 1990). So such results are also possible. " Exception to generalization are common since individual animals do not 'read the book' and follow the disease pattern exactly. For this reason where confidence in the absence of MAP infection is desired , use of two or more different types of tests at the same time is recommended for the best available information" (Collins, 2003b).

Due to complex nature of pathogenesis and epidemiology of MAP infection, diagnosis and control is often difficult but not impossible. Usefulness of different diagnostic assays in different stages of disease varies considerably. A number of diagnostic tests are available that can be used judiciously for diagnosis. Development of new diagnostic assays endowed with desired efficiency can be expected in the future years to come as the scientific knowledge advances in relation to MAP. It is therefore imperative to accept reality of paratuberculosis and ensure the fullest use of available technology in the diagnosis, control and prevention, rather than waiting for technological revolution leading to development of a perfect test.

In this present study the amplification of the IS*900* insertion element was the most specific and sensitive diagnostic detection method (21 per cent), followed by single intradermal Johnin test (5.5 per cent) and acid fast staining detected the

least number of infected animals (3 per cent). From the above result it is concluded that PCR is a better tool in diagnosing *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in goats, when compared to acid fast staining and single intradermal Johnin test, both in organized farm and field level. Single intradermal Johnin test can be graded as second best diagnostic test among the three tests studied, as if has got the added advantage of being less expensive and field oriented than PCR. Acid fast staining of faecal smear is the least reliable technique in diagnosing early stages of paratuberculosis, before frequent high level shedding of organism in advanced stage. Early stage of infection can be better diagnosed by cell mediated immune response using single intradermal Johnin test, before the initiation of faecal shedding of organism. Polymerase chain reaction amplification of MAP is the most reliable technique for identification of infected animals and better suited at any stage of the disease. Polymerase chain reaction examination of faecal sample is the best way to confirm diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* in goats, comparing faecal smear examination by Ziehl-Neelsen acid fast staining and single intradermal Johnin test.

Summary

6. SUMMARY

The study was conducted to compare the efficacy of acid fast staining of faecal smear, single intradermal Johnin test and IS*900* PCR for diagnosis of paratuberculosis in goats. One hundred and fifty goats from University sheep and goat farm, Mannuthy and fifty goats from field were used in this study.

All the animals were subjected to Johnin single intradermal test. The animals were injected with 0.1 ml of Johnin purified protein derivative (PPD) intradermally on the mid neck. Thickness of the skin was measured just before and 72 hours after the injection.

Faecal samples from all the above two hundred goats were collected directly from rectum and were subjected to Ziehl-Neelsen acid fast staining to detect the presence of *Mycobacterium avium* subsp *paratuberculosis* (MAP).

Deoxyribonucleic acid was separated from two hundred faecal samples using QIAamp DNA Stool Mini Kit, and subjected to polymerase chain reaction (PCR) using primers specific for IS*900* and subsequently performed submarine agarose gel electrophoresis to detect amplification of 279 bp bands specific for MAP.

Six goats (3 per cent) were positive for MAP by Ziehl-Neelsen acid fast staining of faecal smear and eleven goats (5.5 per cent) were positive by single intradermal Johnin test and fourty two goats (21 per cent) were positive for paratuberculosis by IS*900* PCR.

For all the three tests only two animals were found positive, all the faecal smear positive animals by acid fast staining were found positive in IS*900* PCR also. None of the animals were found positive in faecal smear and single intradermal Johnin test alone at a single point of time. Two single intradermal Johnin positive animals were found positive by IS*900* PCR.

Results of the present study revealed that IS*900* PCR is superior to single intradermal Johnin test and Ziehl-Neelsen acid fast staining of faecal smear for early diagnosis of paratuberculosis in goats. Single intradermal Johnin test is

graded as the second best test among the three tests compared, capable of detecting early cases, in a cost effective way and its added advantage is ease of application. Examination of faecal smear by acid fast staining is found to be the least dependable technique in diagnosing paratuberculosis in goats. Polymerase chain reaction was significantly different from acid fast staining of faecal smear and single intradermal Johnin test in the present study.

Hence from the observations made in this study, it is concluded that PCR is the best diagnostic tool for early detection of paratuberculosis in goats, for making effective culling and thereby controlling this malady.

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COMPARATIVE EFFICACY OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS IN GOATS

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Abstract of the thesis submitted in partial fulfillment of the requirement for the degree of

Master of Veterinary Science

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ABSTRACT

Paratuberculosis (Johne's disease) is a chronic debilitating infection of goats caused by *Mycobacterium* avium subsp *paratuberculosis* (MAP). Recently the organism was reported to be associated with enteric infection in humans and hence the disease is of public health importance. In the present study comparative efficacy of acid fast staining of faecal smear, single intradermal Johnin test and IS*900* faecal PCR were investigated in two hundred goats for detection of MAP. Single intradermal Johnin test was carried out in two hundred goats. Faecal sample from all two hundred goats subjected to Ziehl-Neelsen acid fast staining and IS*900* PCR for detection of MAP.

Out of one hundred and fifty goats screened for paratuberculosis from University sheep and goat farm, Mannuthy, five animals (3.33 per cent) were found positive by acid fast staining, eight animals (5.3 per cent) were found positive by single intradermal Johnin test and thirty animals (20 per cent) were found positive by IS*900* PCR.

Out of fifty goats screened for paratuberculosis from field, one goat (2 per cent), three goats (6 per cent) and twelve animals (24 per cent) were found positive by Ziehl-Neelsen acid fast staining of faecal smear, single intradermal Johnin test and IS*900* PCR respectively

Results of present study from both farm and field indicate that amplification of the IS*900* insertion element was the most specific and sensitive diagnostic method. The strategic use of PCR can provide a means for early identification of MAP infected goat, thus ensuring their elimination from an infected herd. Advantage of skin test is its ease of application and its low cost. Culling of CMI test positives could be a cost effective means of removing infected animals before they actually start faecal shedding. Ziehl-Neelsen staining had the lowest detection rate of the three tests under investigation and it proved unreliable in diagnosing Johne's disease.