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Research Article

DESCRIPTION OF THE INFECTIOUS STATUS IN MURRAH BUFFALO HERD NATURALLY INFECTED BY *MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS* (MAP) IN TAMILNADU

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ABSTRACT
The surveillance for paratuberculosis in Livestock Research Station (LRS), Kattupakkam revealed 12 sero-reactors in a dairy buffalo herd of 23 animals that had no clinical signs of *Mycobacterium avium subsp paratuberculosis* (MAP) infection. Paratuberculosis has been a clinical problem in sheep and goats for the recent years in this farm. All the 12 buffalos were culled and a thorough investigation of the infection status was conducted by IFN-γ immunoassay, serological assay (ELISA), and pathological and bacteriological examination. By IFN-γ immunoassay, 8 animals proved positive, 3 animals proved weakly positive and whereas by serological assay, all the 12 animals proved positive. Histopathological lesions suggestive of paratuberculosis were diagnosed in 10 animals aged between 6 and 8 years. Seven out of 12 animals of which paratuberculosis were verified by bacteriology had positive sero reaction in the IFN-γ immunoassay. Infection status could be confirmed by isolation of the MAP in 7 out of 12 animals; however the polymorphism of the bacterial isolates could not be established in this study. Eventually, many animals being positive in one or both of the immunological tests it indicative of the herd were heavily infected and maintained in subclinical form of MAP without pronounced clinical signs. In conclusion of this study it emphasized that cross reaction with other Mycobacteria might have been caused some of the immunoreactions in these animals.

***Key Words*:** *Paratuberculosis, Mycobacterium Avium Subsp Paratuberculosis, Ifn-Γ Immunoassay, Elisa and Buffalos*

INTRODUCTION
Paratuberculosis (Johne's disease) is chronic granulomatous enteritis of ruminants. It is caused by *Mycobacterium avium subsp paratuberculosis* (MAP). Most infected animals excrete the bacteria in faeces, months to years before clinical signs of infection develop (Sweeney *et al*., 1992). In India, paratuberculosis has been endemic in animal population, while only sporadic cases have been diagnosed in other livestock species (Singh *et al*., 2007), but economic losses have never been estimated or realized. Disease is usually underreported due to difficulties in diagnosing long pre-clinical phase and is listed B disease (OIE) and has trade restrictions. Malabsorption of nutrients leads to negative energy balance in the body is protein loosing enteropathy of domestic ruminants (Kreeger 1991). From 1997-2010, MAP was confirmed from 98 white cattle and 78 black cattle (Project closure reports, DVEPMD, Madras Veterinary College,). Scheme on surveillance and control programme for bovine paratuberculosis was implemented in Tamilnadu, funded by Government of Tamil Nadu. During the first two years of the programme, samples from cattle, sheep and goats in all the Government farms were examined by serology, histopathology and /or bacteriological culture from faecal samples and/or organs. In total, 1205 animals were examined by serology, where of approximately 9% were positive. However, the infection could only be verified in 12 animals in the Livestock Research Station (TANUVAS). Initially, single intradermal test (SID) and serological examinations were used to screen the herd and on average about 3% of the animals tested were found to be seroreactors. Analysis of data showed that single intradermal test was equally good but the doubtful cases should be tested by Double intradermal (DID) test (Annual

report 1999). These findings might indicate that the infection is more widespread in the animal population than has been assumed during the last one decade. However, sero reactors could be the result of cross-reactions between MAP and other microbes. Such cross-reactions are well known between Mycobacteria (Sohal *et al*., 2007 and OIE 2008).

MAP infection in a herd is a dynamic process, where the infection status is dependant on many factors viz. the number of animals shedding bacteria, number of animals developed protective immune response (CMI) and faecal shedding of bacteria is limited. As Johne's develops (Stage 3 and 4) the protective immune response decline and serum antibody responses (humoral response) develop, but fail to protect against host. Isolation of MAP by cultivation is the definitive method for the detection of an infection in a herd. However, it is well known that animal's mighty be infected without shedding bacteria. Serological, pathological and bacteriological methods have singly or together been used to describe the infection status in naturally infected cattle (Jacobsen *et al*., 2000 and Gasteiner *et al*., 2000) and an IFN-γ test has been evaluated for diagnosis of infection in young cattle (Jungersen *et al*., 2002). However, only few studies that include immunological, pathological and bacteriological analysis of the total cattle population in a herd. The Cattle and Buffalo Breeding Unit, Livestock Research Station were included in the surveillance and control programme for paratuberculosis, 12 out of 62 dairy buffalos were found to have positive seroreactors. Amongst them 8 buffalos had high levels of antibodies, 3 buffalos were culled and histopathological and bacteriological examination revealed paratuberculosis. All the remaining animals were culled one year after paratuberculosis was diagnosed in the herd. The present study is undertaken to investigate thoroughly the infection status in this herd at the time of culling, by the use of IFN-γ immunoassay, ELISA, Bacteriological and pathological examination is under report.

MATERIALS AND METHODS

Farm Management

The study area of Livestock Research Station, Kattupakkam was situated 45 km south of Chennai, Tamilnadu, India. The region is within a semi-agro-ecological zone and is having moderate rainfall with long summer. The cattle, sheep and goats were allowed for grazing on the pastures for 5-8 hours depending on the weather condition. In 2001, paratuberculosis was first diagnosed in a buffalo herd in this farm. The livestock on the farm consisted of 105 (Jersey cross), 62 Murrha buffalos, 273 sheep (Madras Red), 187 goats (Boer x local), 576 pigs (Durac, Landrace, Large White Yorkshire and Desi pigs), 458 rabbits (New Zealand white, Soviet chinchilla) and 76 adult ostriches. During the period 1997-2007 several buffalos showed clinical signs of paratuberculosis and MAP was isolated from 12 buffalos. The cow, sheep and goats were examined for the infection simultaneously and clinical, serological, pathological data are recorded for these animals during this period. The production of infected animal milk was terminated in 2008. The herd followed typical Indian husbandry practices. During the winter seasons (November-February) all the animals were allowed grazing for 2-3 hours/day. The milking animals and the heifers were kept in separate sheds, the fattening bulls and 2-3 month old calves were kept in small pens and protected from the outdoor inclement weather. However, according to observations made by a farm in charge (Veterinarian) the cows, buffalos, sheep and goats were kept on pastures. Occasionally the animals had contact with sheep and goats, wild animals such as deer while grazing on the pastures. The dairy animals in the herd were in good health and had an average milk production. No clinical signs of paratuberculosis were noted in any animals at the time of culling.

Serological examination

Serological examination was performed on 62 animals. The serum samples were tested with commercial enzyme linked immunosorbent assay (ELISA) for antibodies against MAP (Herd check ^{TM}). The testing was performed as per the manufacturer's instruction with s/p (sample to positive) ratio > 0.1 in duplicate. The results were categorized as positive with s/p ratio > 0.3, doubtful with s/p ratio < 0.3 and > 0.2 and negative with s/p ratio < 0.2.

Interferon Gamma Immunoassay

IFN-γ immunoassay was performed on 34 animals as per the procedure of Sohal *et al*. (2007). The results were expressed as OD 450 nm values in paratuberculosis derived purified protein derivative (PPDp) stimulated well minus OD values in control wells. OD values > 0.4 were classified as positive, OD values <0.4 and >0.1 as weakly positive, while OD values below < 0.1 were classified as negative.

Pathological Examinations

A full postmortem examination was performed on three animals in July 2010. The animals were euthanized with intravenous Pentobarbital and the postmortem examination was performed immediately. Tissues from various organs were collected for histopathological examination including sections from the mid and distal jejunum, the ileum and the ileocaecal valve and mesenteric lymph nodes. The rest of the animals (9 animals) were sent to Department of Veterinary pathology, Madras Veterinary College for academic purposes during the same year. The materials was collected for histopathology from each animals at the slaughter; samples from the mid-jejunum, distal jejunum, ileum, ileocaecal valve, proximal colon, a jejunal lymph node and the caecal lymph node. Tissues were fixed in 10% neutral buffered formalin and processed by routine paraffin embedding sections of 2-3µm were cut, mounted and stained with haematoxylineosin (HE) and Ziehl-Neelsen (Zn) method was performed for detection of acid-fast bacilli from 9 of the 12 animals, in addition to serial sections of undeterminable granulomatous lesions in several animals.

Bacteriological Examination

Bacteriological examination was performed on faecal samples and on samples from the ileocaecal valve and the mesenteric lymph nodes (Saxegaard, 1985) from all 12 animals in the herd. Samples were decontaminated with 4% sodium hydroxide and 5% oxalic acid with 0.1% malachite green and inoculated on to selective and non-selective Dubos medium with mycobactin (2µg/ml) and pyruvate (4mg/ml).

RESULTS AND DISCUSSIONS

Results from the serological examination, IFN- assay, bacteriological and pathological examinations are presented in table 1.The present study used a multiple diagnostic tests to confirm that the herd was infected with MAP. The sensitivity and specificity of the diagnostic tests depends among other factors on the prevalence of the MAP infection with in the herd and will thus give different results from herd to herd. However the infection in animals is usually quite easily confirmed by faecal culture and serology. About 65 per cent of the remaining clinically healthy animals in the herd will be infected, but only 2/4 of these will be detected by faecal culture (Whit lock *et al*., 1996). In the present herd no animals showed clinical signs of paratuberculosis, but 8 animals were found to shed bacteria in the faeces (Table 1). There for in this herd of 34 animals, the positive prediction would be about 12 (35.3%) animals were infected. Serology and IFN-γ assay detected 8 positive and 3 weakly positive/doubtful animals in either one or both of the tests indicating that more than half of the herd was infected. This finding is consistent with a buffalo herd heavily infected with MAP, although clinical signs would have been expected particularly in the 8 animals that were 6 years older.

In general, the diagnostic results of the immunological tests showed a weak trend towards younger animals having raised IFN-γ results and older animals having raised serological results. A raise in the cell mediated immune (CMI) response in young animals and in the antibodies in older animals have been a common finding in many paratuberculosis studies. Jacobsen *et al*. (2000) observed the CMI response in cattle shortly after the infection and the high proportion of CMI reactors noticed during the first 2 years of life indicating that the majority of individuals become infected during this period.

Investigations in sheep and goats have shown a relationship between pathological findings and the CMI response (Perez *et al*., 1999) and it has been suggested that the CMI response gives protection against the development of diffuse lesions. Our results indicate that a CMI response persisted in the animals for several years following infection, which possibly explore the limited clinical problems in this herd.

Production of antibodies is often correlated with progression of the infection (Nielsen *et al*., 2002 and OIE 2008) and in our study 8 of 12 buffalos with histopathological lesions had high levels of antibodies

Table 1: Results of tests employed for diagnosis of paratuberculosis

S. No	Animal No.	IFN-γ	ELISA	PCR		SID	Culture	Histology
				Milk	Dung			
1.	Buffalo M1	P	P	P	N	P	P	Occasional acid fast organism in lymhnode
2.	Buffalo M5	P	P	P	N	P	P	Severe granulomatous enteritis
3.	Buffalo M7	P	P	P	N	P	N	occasional acid fast organism in lymhnode
4.	Buffalo M10	P	P	P	P	N	P	No characteristic findings
5.	Buffalo M17	P	P	P	N	N	P	No characteristic findings
6.	Buffalo M12	P	P	P	P	N	N	Numerous acid fast organism in lymhnode
7.	Buffalo M20	P	P	P	P	P	N	Occasional acid fast organism in lymhnode
8.	Buffalo M23	P	P	P	P	N	P	Numerous acid fast organism in lymhnode
9.	Buffalo M28	P	P	N	P	N	N	Moderate acid fast organism in lymhnode
10.	Buffalo M158	N	P	N	P	N	N	Moderate acid fast organism in lymhnode
11.	Buffalo M163	P	P	P	P	P	P	Occasional acid fast organism in lymhnode
12.	Buffalo M169	P	P	P	P	P	P	Numerous acid fast organism in lymhnode

(N- Negative; P-Positive; IFN- γ – Interferon – γ Assay; SID – Single intra dermal test)

and small granulomatous inflammatory lesions in the intestine devoid of demonstrable acid-fast bacilli or foreign material and could there fore have been due to MAP infection (Figure 1). This type of lesion was not found in seropositive than in seronegative animals and many seropositive animals had no gross and histopathological lesion indicative of paratuberculosis. More obviously tissue sampling for both histopathological and bacteriology may have confirmed infection in additional animals, since discrete sub clinical lesions can be widely distributed throughout the intestinal tract and mesenteric lymph node is very well corroborated with the report of OIE (2008).

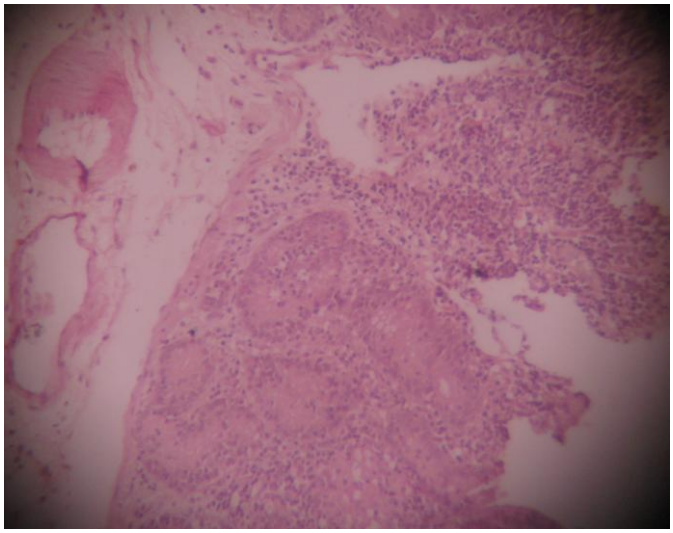

Figure 1: Histopathology of intestine showing thickening of the mucosa with mononuclear infiltration (x400)

In the present study, six confirmed positive animals were 4-6 year old and the result revealed that animals up to 4 years of age the IFN-γ immunoassay would appear to be the relevant screening test, while a test measuring antibodies would be preferable in animals from 3 years and above, the age of the animals can have an impact on the IFN-γ results. False positive reactions have been observed when the IFN-γ test has been applied in calves less than 1.5 years of age (Jungersen *et al*., 2002). Further more, cross reactions with other mycobacterium are common and reducing the specificity of both serological and IFN- γ assays (McDonald *et al*., 1999 and OIE 2008). These cross reacting mycobacteria are common in the environment and could well have caused some of the immuno-reactions in animals in the present study. Results from the surveillance and control programme for bovine paratuberculosis have shown that 9% of buffalo cattle are seroreactors. A follow up study of these seropositive buffalo cattle has shown that the reactions were false positive and were probably caused by environmental bacteria (Fredriksen *et al*., 2004). Paratuberculosis had been a clinical problem in sheep and goat in this farm for the past 3 years before the present study was conducted and the infection might well have existed in cattle in a sub clinical form. The present study shows that the infection might be sub clinical in cattle herd and may be overlooked if immunological, pathological and bacteriological investigation is not performed.

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