Haryana Vet. (Dec., 2017) **56 (2),** 179-183

Research Article

METABOLIC ADJUSTMENTS AS SIGNATURES FOR PATHOGENESIS AND DIAGNOSIS OF JOHNE'S DISEASE IN GOATS

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ABSTRACT

Study is based on 37 goats that were screened for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection, the cause of Johne's disease (JD) in domestic livestock. Status of disease in goats was determined using physical profile and screening by 3 diagnostic tests (microscopy, indigenous ELISA and fecal PCR). Various biochemical parameters were estimated in the serum samples driven from both infected (MAP positive), weak and non-infected (MAP negative) and healthy goats. Since JD was endemic in domestic goat population, at sampling time, goats were in different stages of incubating disease. Study reports haematological and biochemical profiles of goats in different stages of disease. Screening of blood and serum samples was fruitfully employed for the diagnosis of JD**.** Significantly higher levels of chloride, creatinine, total protein, triglycerides and cholesterol were recorded. Metabolites of MAP, such as triglycerides, creatinine and electrolyte like chloride could be useful signature to explore further their significance in pathogenesis and diagnosis of Johne's disease in domestic livestock.

Key words: Johne's disease, Paratuberculosis, *Mycobacterium avium* subspecies *paratuberculosis*, metabolic profiling

Johne's disease (JD) is chronic granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) affecting both animals and human population. As per OIE, JD is a notifiable disease. Major concern with MAP infection is chronic and insidious nature of disease, non-specific symptoms, shedding of bacilli in milk and feces is variable in the sub clinical stage and development of detectable immune response may take long time to generate.

Johne's being an incurable disease of animals and human beings, the 'Test and slaughter' strategy adopted for reducing the bio-load of MAP in goat flocks located at CIRG, Makhdoom for past 37 years, has not worked. This is mainly due to the fact that MAP is transmitted to next generation through semen, during pregnancy, by suckling of milk and colostrum. Ability to diagnose JD in early stages of infection or disease, that is before animals turn into sub-clinical shedders) and use of effective vaccine are critical for the control of JD in herds / flocks. Using multi-drug chemo-therapy for long time may help in remission of lesions and disease but is not cost effective. Therefore, in the absence of effective control regimen, endemically infected animals continue to infect new herd mates goats, thus making eradication of disease impossible. Early diagnosis is critical for the management and control of disease in herds and flocks.

Control of JD is not only important but highly critical for the economic viability of livestock enterprise / industry. To prevent human infection, it will be essential to reduce or stop shedding of MAP in milk, thereby preventing transmission to human population through regular consumption of milk and milk products. Non-availability of sensitive and specific test kits is the major limitation in diagnosis particularly during early stages of infection. Good indigenous vaccines with both prophylactic and therapeutic properties though recently developed (Singh *et al*., 2007), but is not yet available commercially. Imported vaccines based on MAP strains (not reported from India), are not only less effective but also not cost effective as compared to 'indigenous vaccine' based on native strain of MAP (Singh *et al.,* 2007). Efficacy of cattle based imported vaccines in goats is questionable. Till control of JD using vaccine is not Government sponsored program, goat owners being poor may not be able to afford the vaccination. Vaccinating huge (>500 million) population of livestock and their progenies may not be a simple task in India, where animals lack proper identification. Therefore alternate systems have to be identified for the control of this devastating disease.

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Country is slowly loosing the rich diversity of germ-plasm in-terms of livestock breeds due to JD. Disease being endemic in native livestock and a spectral disease, hence at any given point of time, animals will be different stages of incubating disease. Comparative profiling of blood samples from JD positive and negative goats may help in establishing panel of diagnostic markers for the early detection of infection.

MATERIALS AND METHODS

Sampling and Testing of Samples: Blood samples were collected from female goatherds (n=17+20) located at Central Institute for Research on Goats (CIRG), Makhdoom, Uttar Pradesh and Kiratpur, Madhya Pradesh. Adult goats of Barbari, Sirohi and Jakhrana breeds were sampled. Sampled goats were in different physical profile (extremely weak, weak, normal and healthy) (Singh *et al*., 2014). Serum samples were stored at -20^{0}C till further analysis. Serum samples were screened by indigenous ELISA kit to estimate sero-status (negative or positive) of JD. Blood and feces were screened by PCR and microscopy for confirmation of JD (Singh *et al*., 2013). Goats positive in three tests twice at one month apart were considered positive for MAP infection. Serum samples from apparently healthy goats negative in three tests were taken as negative controls. Positive and negative serum samples were screened by semi-automatic and automatic 'Biochemical Analyzer' for chloride, bilirubin, albumin, phosphorus, creatinine, total protein, magnesium, calcium, urea, cholesterol, alkaline phosphatase, uric acid, SGPT, SGOT, Gamma glutamyl-transferase and triglycerides (Table 1). Data was analyzed statistically by One way analysis of variance and Dunnett's Post hoc Test using SPSS version (Snedecor and Cochran, 1994) to detect significant difference between positive and negative goats.

Polymerase Chain Reaction: DNA isolation from blood and PCR was done as per method van Embdem *et al*. (1993) and van Soolingen *et al*. (1993) with some modifications (Singh *et al*., 2010). DNA isolation from fecal samples was done as per the method of Singh *et al*. (2013). Primers sequences used were as per Millar *et al*. (1995). Forward primer- P90B: 5'-GAA GGG TGT TCG GGGCCGTCG CTT AGG -3' and Reverse primer- P91B: 5'-GGC GTT GAG GTC GATCGC CCA CGT GAC -3' were used in this study. The resulting gel picture after IS*900* PCR has been depicted in Fig.1.

Serological Tests: Goats were screened using 'Indigenous ELISA kit' as per Singh *et al.* (2007). OD values were converted into S/P ratio and corresponding status of JD in animals (Collins, 2002): Following Table 2 depicts the sample to positive ratios (S/P) and status of Johne's disease in the host.

Microscopy: Two gram of feces was homogenized with sterilized normal saline solution and finely grounded

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

Table 1

Profile of the serum samples, positive and negative controls supplied to LUVAS, Hisar by CIRG, Makhdoom

Sn.	Goats						Goats					
	Positive control			Negative Control			Positive control			Negative Control		
	Sample Id		Sex, Physical Status & place	Sample Id		Sex, Physical Status & place	Sample Id		Sex, Physical Status & place	Sample Id		Sex, Physical Status & place
1	GP1	27	F, 3+, Kiratpur	GN1	JK995	F, 4+, CIRG	GP1	ND 301	F, 2+, CIRG	GN1	ND 299	F, 4+,CIRG
2	GP2	10443	F, 2+, CIRG	GN2	5	F, 3+, Kiratpur	GP2	ND 305	F, 3+, CIRG	GN2	ND 304	F, 4+,CIRG
3	GP3	9376	F, 3+, CIRG	GN3	J7955	F, 4+, CIRG	GP3	ND 308	F, 2+, CIRG	GN3	ND 312	F, 3+,CIRG
4	GP4	G15	F, 2+, Kiratpur	GN4	G1	F, 3+, Kiratpur	GP4	ND 309	F, 3+, CIRG	GN4	ND 310	F, 4+,CIRG
5	GP5	2	F, 3+, Kiratpur	GN5	B	F, 4+, Kiratpur	GP5	988	F, 2+, CIRG	GN5	ND 306	F, 3+,CIRG
6	GP6	G3	F, 2+, Kiratpur	GN6	74	F, 4+, Kiratpur	GP6	137	F, 2+, CIRG	GN6	ND 297	F, 4+,CIRG
7	GP7	1712	F, 2+, Kiratpur	GN7	4	F, 4+, Kiratpur	GP7	150	F, 3+, CIRG	GN7	ND 221	F, 3+,CIRG
8	GP8	9	F, 3+, Kiratpur	GN8	13	F,4+, Kiratpur	GP8	851	F, 2+, CIRG	GN8	ND 226	F, 3+,CIRG
9	GP9	13	F, 3+, Kiratpur	GN9	5	F, 3+, Kiratpur	GP9	160	F, 2+, CIRG			
10	GP10	15	F, 2+, Kiratp.0ur	GN10	1	F, 4+, Kiratpur						

Kiratpur - Kiratpur, MP; CIRG - CIRG, Makhdoom; 4+ = Healthy; 3+=Weak; 2+ = Weak and Suspected for JD; 1+ =Clinical cases of JD; J - Jamunapari (CIRG); Jk - Jakhrana (CIRG); Barbari (CIRG); Sirohi (CIRG); F=Female

Table 2
S/P ratios and corresponding status of Johne's disease

S. No.	Calculated value of S/P Ratio	Johne's disease status in animals	Status
1	0.00 – 0.09	Negative (N)	Negative
2	0.10 – 0.24	Suspected or borderline (S)	
3	0.25 – 0.39	Low positive (LP)	
4	0.40 – 0.99	Positive (P)	Positive
5	1.00 – 10.0	Strong positive (SP)	

*Animals in Positive and Strong Positive categories of S/P ratio were taken as 'Positive'.

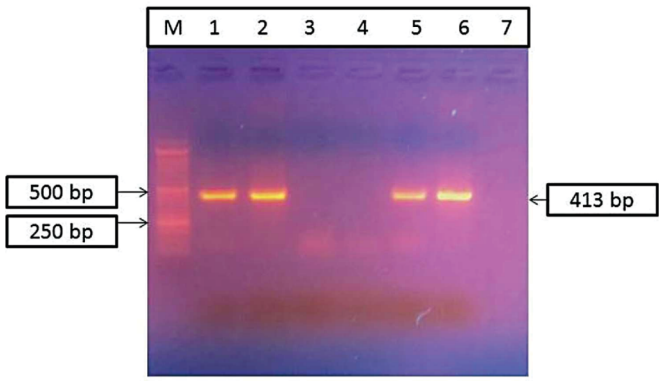

Fig. 1 : MAP IS900 PCR: Lane M. 50bp ladder (Hi-Media), Lane 1: Positive control (MAP Indian Bison type DNA), lane 2 to 7: Test Samples (Lane 2, 5 and 6 are positive and 3, 4 and 7 are negative)

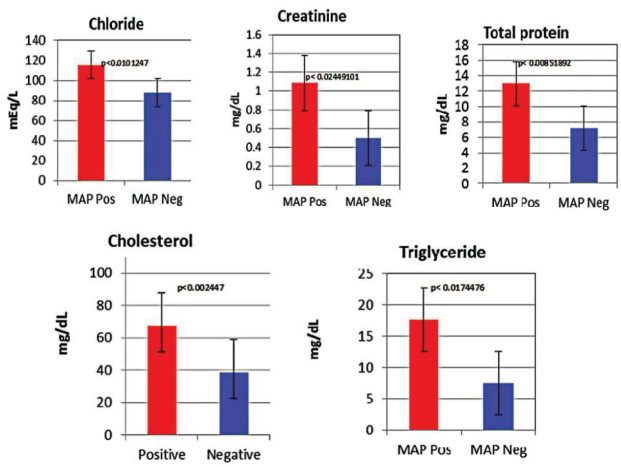

Fig. 2 : Screening of serum samples of goat affected with Mycobacterium avium subspecies paratuberculosis for biochemical parameters. Serum concentrations of: A= Chloride; B=Total Protein; C=Creatinine; D=Cholesterol and E=Triglycerides in MAP positive and negative animals

in pestle and mortar. Paste was diluted with 7-8mL of sterilized NSS and transferred in 15 mL centrifuge tubes. Tubes were centrifuged at 4000rpm for 45 min. Supernatant was decanted and a thin smear was made semi solid middle layer. Slides were heat fixed and stained with Ziehl Neelsen's stain and visualized under microscope for pink colored short rods (Singh *et al*., 2010; Singh *et al*., 2013).

RESULTS AND DISCUSSION

Mean values of metabolic parameters are presented in Fig. 2 (A to E). Significantly higher levels of chloride (A), creatinine (B), total protein (C), cholesterol (D) and triglycerides (E) were found in MAP positive animals as compared to the MAP negative animals.

Hyper-triglyceridemic serum has been shown to enhance *Mycobacterium avium* replication at least in human macrophages (Douvas *et al*., 1994). It is also shown that uptake and trafficking of MAP in human cells are cholesterol dependent and these bacilli localize in cholesterol-rich compartments (Keown *et al*., 2012). During lipid profiling of MAP strains, Singh *et al*. (2000) reported very high contents of triglycerides, while studying lipid profile of native MAP strains for establishing Mycolic acid patterns.

Existing literature suggests that MAP infection is associated with hyper-lipidimia but it is still unclear whether this is a mycobacterial strategy to evade host immune system or involvement of other mechanisms. Significantly higher levels of total cholesterol and triglycerides were observed in the JD positive (confirmed) cases. Data suggests that lipid profile changes accompany JD, some of which may indicate severity and guide therapy. Hyper-cholesterolemia has also been reported in acute and chronic nephritis, chronic nephrosis, cholelithiasis and biliary obstruction (Kaneko *et al*., 2008). Thus our current data warrants a more detailed study on lipid profiling in MAP positive and negative animals.

Our findings of significantly increased total protein content in MAP positive goats is consistent with other

studies suggesting that progress of infection is usually associated with marked changes in serum proteins. There may be increase in percentage of total protein during some stage of infection. In albumin-globulin ratio there is usually a change with an increase in total globulins (Hurwitz and Meyer, 1916). There was no significant difference in albumin levels of positive and negative goats. Gamma globulin production has been shown to increase in response to antigens (infections, suppurative diseases, chronic infections etc.) (Kaneko *et al*., 2008). Globulin levels will be worth measuring in these animals.

To our surprise significant increase in serum creatinine levels in MAP positive goats was observed. It may be possible that MAP infection causes secondary renal disease which could result in higher serum creatinine levels. Solak *et al*. (2013) also reported similar findings. Over production of local immuno-globulins in the intestinal mucosa has been reported in MAP infection. Excessive circulating antibodies participate in the pathogenesis of disease along with formation of immune complexes (Momotani *et al*., 1986). Glomerulo-nephritis or fibrinoid and amyloid-like deposits in small vessels of various organs in caprine JD strongly suggest participation of immune complexes in pathogenesis (Sato *et al*., 1968). Question whether creatinine levels could aid as diagnostic parameter for JD requires further investigation with on large number of animals and in different species. Vice versa, creatinine levels could be plotted against MAP infection and level of MAP infection can be used to indicate creatinine levels in blood.

Kidneys are responsible for the maintenance of total body chloride balance either by the addition of excess chloride to the extra cellular compartment or by the loss of water from this compartment, and vice versa. Several conditions are associated with hyper-chloremia (Walker, 1990). Observation of higher serum concentrations in MAP positive animals might be associated with either of these conditions which could be evaluated by investigating increased number of animals and including various animal species that are suffering with MAP infection.

REFERENCES

Collins, M.T. (2002). Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using Likelihood Ratios. *Clin. Vaccine Immunol.* **9**: 1367-1371.

Douvas, G.S., May, M.H., Pearson, J.R., Lam, E., Miller, L. and Tsuchida, N. (1994). Hypertriglyceridemic serum, very low density lipoprotein, and iron enhance *Mycobacterium avium* replication in human macrophages. *J. Infect. Dis.* **170**: 1248-55.

Hurwitz, S.H. and Meyer, K.F. (1916). Studies on the blood proteins: The serum globulins in bacterial infection and immunity. *J. Exp. Med.* **24**: 515-546.

Kaneko, J.J., Harvey, J.W. and Bruss, M.L. (2008). Clinical Biochemistry of Domestic Animals. Burlington, MA: Elsevier.

Keown, D.A., Collings, D.A. and Keenan, J.I. (2012). Uptake and persistence of *Mycobacterium avium* subsp. p*aratuberculosis* in human monocytes. *Infect. Immun.* **80**: 3768-3775.

Millar, D.S., Withey, S.J., Tizard, M.L.V., Ford, J.G. and Hermon-Taylor, J. (1995). Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*. *Analyt. Biochem.* **226**: 325-330.

Momotani, E., Ishikawa, Y. and Yoshino, T. (1986). Immuno-histochemical distribution of immunoglobulin and secretory component in the ileum of normal and paratuberculosis-infected cattle. *J. Comp. Pathol.* **96**: 659-669.

Sato, H., Nakamatsu, M. and Fujimoto, Y. (1968). The pathological study of paratuberculosis in goats, centered around the formation of remote lesions. *Japanese J. Vet. Res.* **16**: 103-119.

Singh, S.V., Singh, P.P., Singh, N. and Gupta, V.K. (2000). Characterization of lipid pattern of *Mycobacterium paratuberculosis* isolates from goats and sheep. *Ind. J. Anim. Sci.* **70**: 899-903.

Singh S.V., Singh P.K., Singh A.V., Sohal J.S., Gupta V.K. and Vihan V.S. (2007). Comparative efficacy of an indigenous 'inactivated vaccine' using highly pathogenic field strain of *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' with a commercial vaccine for the control of Capri-paratuberculosis in India. *Vaccine* **25**: 7102–7110. doi: 10.1016/j.vaccine.2007.07.054.

Singh, S. V., Singh, A. V., Singh, P. K., Sohal, J. S. and Singh, N. P. (2007). Evaluation of an indigenous ELISA for diagnosis of Johne's disease and its comparison with commercial kits. *Indian J. Microbiol.* **47**: 251-258.

Singh, P.K., Singh, S.V., Kumar, H., Sohal, J.S. and Singh. A.V. (2010). Diagnostic application of IS*900* PCR using blood as a source sample for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in early and subclinical cases of caprine paratuberculosis. *Vet. Med. Int.* 2010: 748621. doi: 10.4061/2010/748621.

Singh, S.V., Singh, P.K., Gupta, S., Chaubey, K.K., Singh, B., Kumar, A., Singh, A.V. and Kumar, N. (2013). Comparison of microscopy and blood-PCR for the diagnosis of clinical Johne's disease in domestic ruminants. *Iran J. Vet. Res.* **14**: 345-349.

Singh, S.V., Singh, P.K., Singh, A.V., Sohal, J.S., Kumar, N., Chaubey, K.K., Gupta, S., Kumar, A., Bhatia, A.K., Srivastav, A.K. and Dhama, K. (2013). Bio-load and bio-type profiles of *Mycobacterium avium* subspecies *paratuberculosis* infection in the farm and farmer's herds/flocks of domestic livestock: A 28 years study (1985-2013). *Transboundary Emerg. Dis.* **61**: 43-55.

Snedecor G.W. and Cochran, W.G. 1994. Statistical Methods. (8th edn.). Oxford & IBH Publishing Co., Calcutta, India.

Solak, Y., Gaipov, A., Anil, M., Atalay, H., Ozbek, O., Turkmen, K., Polat, I. and Turk, S. (2013). Glomerulonephritis associated with tuberculosis: A case report and literature review. *The Kaohsiung J. Med. Sci.* **29**: 337-342.

van Embden, J. D., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B. and Shinnick, T. M. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* **31(2)**: 406-409.

van Soolingen, D., Hermans, P.W., de Haas, P.E., Soll, D.R. and van Embden, J.D. (1991). Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol.* **29**: 2578.

Walker, H.K., Hall, W.D. and Hurst, J.W. (1990). Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edn. Boston: Butterworths. Chapter 197 Serum Chloride, table 197.2.