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Antibodies against *Mycobacterium avium subspecies paratuberculosis* in Cattle of Indore District in Madhya Pradesh

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ABSTRACT

Background: Paratuberculosis or Johne's disease in domestic livestock population is caused by the bacteria, *Mycobacterium avium subspecies paratuberculosis*. The disease in cattle is characterized by chronic granulomatous enteritis leading to diarrhoea (intermittent or continuous) followed by weakness and emaciation in the affected animals. This research aimed to test cattle population, serologically, for detecting level of *Mycobacterium avium subspecies paratuberculosis* infection.

Methods: In the present study, a total of 180 serum samples from individual cattle of Indore district in Madhya Pradesh tested in a serological test, indigenous enzyme linked immunosorbent assay for detection of antibodies against *Mycobacterium avium subspecies paratuberculosis*.

Result: A total of 49 cattle (27.2%) were found positive in the test for the bio-presence of *Mycobacterium avium subspecies paratuberculosis* infection.

Key words: Cattle, Indirect enzyme linked immunosorbent assay, Paratuberculosis.

INTRODUCTION

Paratuberculosis or Johne's disease (JD) is caused by the bacterial agent *Mycobacterium avium subspecies paratuberculosis* (MAP). The disease is characterized by a long-lasting enteritis of large ruminants (Hassan *et al.*, 2019). Besides infecting sheep and goats, it also infects wild ruminants (bison, blue bulls, deer) and human beings (Radostits *et al.*, 2000; Biswal *et al.*, 2020). The 20th livestock census of India recorded a total livestock population of 536.76 million (Cattle-193.46 million (36.04%), Goats-148.88 million (27.74%), Buffalo-109.85 million (20.47%), Sheep-74.26 million (13.83%), Pigs-9.06 million (1.69%) and others i.e. Mithun, Yaks, Horses, Ponies, Mules, Donkeys and Camels (0.23%). Cattle (indigenous, exotic and crossbred) are used in producing milk and utilised for draft purposes in the field and for transportation in the country. Female cattle (cow population-145.91 million) were increased by 18.6% over previous census (122.9 million) indicating increased interest of livestock owners in rearing cattle for milk (VIKASPEDIA, 2021). Milk production in Madhya Pradesh stood at 17.2 billion litres. Cow milk dominated the total milk production in the region in 2019. Dairy market in the state is separated into 18 different major product segments (raw, fermented, value added products from milk) (IMARC, 2019).

So, regular testing of livestock population for monitoring their health status is essential to prevent and control animal infectious diseases and thereby contain losses to dairy sector. Paratuberculosis is diagnosed tentatively clinically (history of chronic diarrhoea that is generally untreatable and extreme emaciation in affected bovines) and also definitively by laboratory diagnostic methods such as acid fast staining (AFS) of faecal material and further confirmation by agent isolation from faeces of infected animal, serological testing by using agar gel immunodiffusion (AGID), complement fixation test (CFT), Enzyme linked immunosorbent assay (ELISA), fluorescent antibody technique (FAT), dot-ELISA and molecular methods such as polymerase chain reaction (PCR). Rectal biopsy or scraping and lymph node

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biopsy followed by AFS, intradermal johnin and intravenous johnin testing are also conducted for the diagnosis (Singh *et al.*, 2016b; Chakrabarti, 2003). In the present investigation, indigenously developed ELISA is used for screening serum samples of cattle to evaluate antibody response against MAP infection.

MATERIALS AND METHODS

Geographic location

Indore district (latitude-22°39′59.99″ N 75°44′59.99 E) is one of the important districts of Madhya Pradesh state in India. It lies in an average altitude of 550 meter above sea level. The district is located in semi-arid zone towards the southern edge of Malwa. The region (predominantly has black soil) lies in rain-shadow area of Western Ghats (Kawadia and Tiwari, 2017). It receives majority of its rains through South-West monsoon. Madhya Pradesh is landlocked and has no international border. It is surrounded by Uttar Pradesh, Chhattisgarh, Maharashtra, Gujarat and Rajasthan (Britannica, 2020).

Serum samples

Blood samples from 180 cattle (Crossbred, non-descript) of Indore and adjacent area were collected randomly irrespective of their clinical status for Paratuberculosis infection and separated serum stored in the deep freezer (-40°C) in the Microbiology Department. These serum samples were transported to Central Institute for Research on Goats (CIRG), Farah under cold chain conditions and stored at deep freezing conditions (-20°C). The testing was done during the year 2016-2018.

Indigenous ELISA

The indigenous ELISA (iELISA) for detection of serum antibodies against Johne's disease or MAP developed in the Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats (CIRG), Farah was employed in the present investigation and briefly described (Audarya *et al.*, 2018).

Antigen preparation

Antigen was prepared from native highly pathogenic field isolates of MAP (S5) recovered from a terminal case (in lateral recumbency due to weakness and debility) of Johne's disease in a Jamunapari goat (which later succumbed and died) located at CIRG farm herds. Antigen was prepared from this S5 'Bison type' strain MAP at 16th passage level. Growth was inactivated (72°C for 2 hours) and pelleted at 10,000 g for 20 min. at 40°C. Pellet was given 3 washing in 0.01 M phosphate buffered saline (PBS) pH 7.2. After 3rd washing pellet was suspended in sterilized normal saline solution in ratio of 0.2 g of wet cell weight/ml, in 30 ml and treated to ultrasonic disruption (sonication) at 100 watts (15 Hz) for 20 min. in ice slurry giving 20 cycles of 30 seconds and 30 seconds rest after each cycle. The sonicate was centrifuged (10,000 rpm for 30 min at 4°C), dispensed and stored at -20°C. Protein concentration was also estimated (Lowry *et al.*, 1951; Sevilla *et al.*, 2005; Personal communication with R.J. Whittington).

Coating of microplates with antigen

Lysate (sonicated whole cell) was centrifuged, standardized and used in coating. 100 µl of working antigen (at 0.1 µg/well of microtiter plate) was added in each well of microtiter plate. Antigen added plates were placed at -4°C for overnight.

Blocking of wells

Overnight antigen coated plates were brought to room temperature and washed once with 1×PBS + 0.05% Tween 20. Wells of the plates were blocked with skimmed milk (100 µl of 3%, in 1×PBS) and plates were incubated (37°C for 1 h).

Testing of serum samples by iELISA

After blocking plates were brought to room temperature and washed thrice with 1×PBST. 1:50 dilution serum samples were used. Thereafter 100 µl of diluted anti-species horseradish peroxidase conjugate (Sigma) (1:5000 dilution) was added and incubated for 1 h at 37°C. On completion of incubation, plates were washed four times with 1× PBST. Thereafter, in each well 100 µl of substrate was added o-phenylenediamine dihydrochloride (OPD) at 5 mg/plate concentration in substrate buffer, pH-5) and plates were incubated at room temperature in the dark for 3-5 min. Culture positive and negative samples of animals were used as positive and negative controls, respectively. Blank was also run. ELISA reader was used to record absorbance at 450 nm.

Interpretation

The following formula was used to transform Optical densities (OD) to sample to positive ratios (S/P ratio) (OD value of test serum sample - OD value of negative control)/ (OD value of positive control - OD value of negative control). Thereafter as per the S/P ratio, if S/P ratio was in between 0.00-0.09- animals were negative, 0.10-0.24- suspected, 0.25-0.39- low positive, 0.40-0.99- positive and strong positive (1.0-10.00) for Johne's disease status. For bio-presence of MAP infection, those animals falling in 0.40 to 10.00 were considered positive (Collins, 2002).

RESULTS AND DISCUSSION

OD values recorded (after testing cattle serum samples in iELISA for the presence of antibodies to MAP infection) were used to calculate S/P ratio. Of the total 180 cattle, 2, 47, 22, 30 and 79 demonstrated S/P ratio in between 1-10, 0.40-0.99, 0.25-0.39, 0.10-0.24 and 0.00-0.09, respectively. Results of the investigation are presented in Table 1, 2 and 3.

Johne's disease is caused by *Mycobacterium avium subspecies paratuberculosis* in large ruminants. It causes chronic progressive diarrhea in cattle and buffalo population.

Table 1: Cattle serum samples tested low positive, positive, suspected, strong positive in indigenous enzyme linked immunosorbent assay for MAP infection.

Sample	S/P ratio	Sample	S/P ratio	Sample	S/P ratio	Sample	S/P ratio
Low positive	N=22						
3	0.262687	19	0.537313	85	0.453731	50	0.107463
6	0.301493	22	0.516418	86	0.501493	52	0.238806
10	0.289552	30	0.585075	88	0.8	59	0.110448
16	0.355224	32	0.540299	92	0.499244	97	0.111952
21	0.253731	33	0.659701	94	0.499244	99	0.199697
23	0.289552	39	0.438806	95	0.711044	101	0.133132
42	0.358209	43	0.453731	100	0.532526	115	0.14826
45	0.250746	44	0.432836	103	0.599092	117	0.118003
48	0.337313	46	0.450746	104	0.559758	124	0.214826
53	0.298507	47	0.528358	112	0.744327	125	0.121029
57	0.391045	51	0.441791	113	0.426626	126	0.136157
60	0.298507	55	0.486567	114	0.456884	128	0.239032
62	0.391045	56	0.456716	120	0.487141	129	0.19062
70	0.295522	58	0.626866	121	0.459909	132	0.130106
79	0.274627	61	0.710448	152	0.459909	137	0.151286
87	0.313433	63	0.429851	165	0.780635	143	0.130106
105	0.329803	64	0.447761	173	0.402421	149	0.118003
109	0.299546	65	0.570149	174	0.420575	156	0.111952
110	0.293495	67	0.731343	Suspected	N=30	160	0.187595
123	0.293495	68	0.656716	4	0.101493	164	0.114977
127	0.387292	69	0.465672	5	0.232836	166	0.130106
145	0.378215	71	0.507463	11	0.185075	175	0.229955
Positive	N=47	75	0.447761	13	0.18806	180	0.166415
8	0.650746	76	0.444776	31	0.158209	Strong positive	N=2
14	0.480597	77	0.441791	34	0.235821	7	1.044776
15	0.961194	80	0.522388	40	0.176119	24	1.062687

Table 2: Cattle serum samples tested negative in indigenous enzyme linked immunosorbent assay for MAP infection.

Sample	S/P ratio	Sample	S/P ratio	Sample	S/P ratio	Sample	S/P ratio
Negative	N=79	66	-0.05373	116	0.051437	151	0.045386
1	-0.04776	72	-0.0149	118	0.08472	153	0
2	0.089552	73	0.071642	119	0.033283	154	0.006051
9	-0.01791	74	0.01791	122	0.072617	155	0.099849
12	0.020896	78	-0.00597	130	0.08472	157	0.051437
17	-0.05373	81	-0.03582	131	0.018154	158	0.093797
18	0.068657	82	-0.02985	133	0.078669	159	0.066566
20	0.089552	83	-0.0209	134	-0.03328	161	0.024206
25	0.01194	84	-0.06866	135	0.051437	162	0.039334
26	0.056716	89	-0.04478	136	0.072617	163	0.048411
27	0.038806	90	-0.01194	138	0.04236	167	0.057489
28	0.047761	91	-0.0121	139	-0.02421	168	0.045386
29	0.080597	93	0.08472	140	0.066566	169	0.036309
35	-0.02388	96	-0.02421	141	0.054463	170	-0.0121
36	-0.00597	98	0.048411	142	0.030257	171	-0.04841
37	0.008955	102	-0.06051	144	0.087746	172	0.006051
38	0.01194	106	0.04236	146	0.027231	176	-0.00605
41	-0.02388	107	-0.01513	147	0.027231	177	0.024206
49	0.023881	108	0.093797	148	0.075643	178	0.054463
54	0.089552	111	0.036309	150	-0.01513	179	0.009077

Table 3: Cattle serum samples divided into different groups according to the outcome of test results for bio-presence of MAP infection.

Outcome	S/P ratio	Number of samples	Percentage
Strong positive	1-10	2	1.11
Positive	0.40-0.99	47	26.11
Low positive	0.25-0.39	22	12.22
Suspected	0.10-0.24	30	16.67
Negative	0.00-0.09	79	43.89

It is also thought to be associated with Crohn's disease and producing cancer in human beings (Pierce, 2018). The disease in large ruminants is characterized by chronic enteritis followed by emaciation. Economic impact of paratuberculosis in cattle was well documented. To reduce the economic impact of paratuberculosis, it is inevitable to know the degree of the MAP infection in cattle for implementing cost-effective disease prevention and control measures (Hasonova and Pavlik, 2006; Garcia and Shallo, 2015). In India MAP infection was reported in livestock population (Audarya *et al.*, 2013; Audarya *et al.*, 2016; Matoli *et al.*, 2018; Sharma *et al.*, 2020). The selection of the diagnostic test for Paratuberculosis was based on many factors (Salem *et al.*, 2013). Various serological and molecular methods have been used for the diagnosis of Paratuberculosis in India and abroad (Gumussoy *et al.*, 2015; Abdelaal *et al.*, 2019).

Hence, in the present investigation, 180 serum samples from cattle from Indore district of Madhya Pradesh in India were investigated for bio-presence of MAP infection in iELISA developed at CIRG. Previously, serological positive percentages of 15.14% and 37.7% in ELISA were detected in cattle from Karnataka and West Bengal in India (Gupta *et al.*, 2012; Bhutediya *et al.*, 2017). In the present study, a total of 49 (27.22%) cattle tested positive for bio-presence of MAP infection. Breed wise susceptibility for MAP infection was not widely studied. In Pakistan, in Sahiwal cattle breed 3-18 times higher chances of disease was found compared to Cholistani breed of cattle (Hussain *et al.*, 2018). Majority of the cattle investigated in the present study were either crossbred or non-descript adult animals so it was unable to throw light on the susceptibility of specific cattle breed and also risk factors associated with sero-positivity to JD (Sun *et al.*, 2015; Garg *et al.*, 2016). However, in future such studies can be planned in the state of Madhya Pradesh where 3 cattle breeds (Malwi, Kenkatha and Nimari), crossbred and non-descript indigenous cattle are reared for milch and draft purposes.

Bio-load of MAP in the livestock population of India is very high (23% to 43%). Large ruminants had higher bio-load (36%-43%) of MAP than small ruminants (23%-41%). Among ruminants, cattle have highest bio-load of MAP and goats have the lowest (Chaubey *et al.*, 2017). MAP causes chronic infection in cattle and also exhibit latency (Nielsen *et al.*, 2013). Cattle provides a significant proportion of milk produced in India. MAP is an important food borne pathogen in India. MAP organisms are shed in the milk of infected cattle too. MAP may also provide foundation for establishing diabetes (Singh *et al.*, 2014). Besides, being in contact with infected cattle herds and consumption of MAP contaminated milk or products made from such milk, humans may catch infection from showers and river aerosols too (Rhodes *et al.*, 2014). However, in cattle, neonates and young animals are infected primarily by the fecal-oral route (Rathnaiah *et al.*, 2017). In India, presence of MAP was reported in various species of animals and their products, human beings and environmental resources from 1960 to till date (Singh *et al.*, 2016a). The results of the present study indicated bio-presence of MAP infection (27.2%) amongst cattle population of the Indore district. The findings of the study will be helpful in implementing prevention and control strategies against Paratuberculosis in Madhya Pradesh (Boschiroli and Thorel, 2010; Geraghty *et al.*, 2014; Whittington *et al.*, 2019). Madhya Pradesh has shared border with many states. So, animal certification and transportation must be regulated with strict measures regarding movement of animals for trade, fairs and other purposes.

Elimination of the infectious agent from the farm by test and slaughter or culling of positive cattle is not practiced in most of the cases due to economical and ethical reasons in India. Hence, disease transmission from infected animals to susceptible young ones must be stopped by using appropriate management practices (such as vaccination of healthy cattle, better hygiene at farms, segregation and treatment of affected cattle and disposal of the contaminate waste) to empower livestock owners (Pradere, 2014). Present vaccine available in India against Paratuberculosis (Bio-JD, having both preventive and prophylactic value) is an inactivated vaccine but it is not that popular amongst livestock owners due to their unawareness about the disease. India has continuously implemented vaccination programme against Foot-and-Mouth disease. Very recently vaccination against Brucellosis is also envisioned for susceptible livestock. Considering the widespread occurrence of Paratuberculosis in India and its economic implications to the livestock owners and industry, regular sero-surveillance campaigns and agent isolation and nationwide Paratuberculosis control strategies like immunizing susceptible cattle/livestock population against the disease in India as well as in the state of Madhya Pradesh is the need of the present times.

CONCLUSION

In the present investigation, indigenously developed ELISA was used for screening serum samples of cattle to evaluate antibody response against MAP infection. The results of the study indicate 27.22% bio-presence of MAP infection in cattle population in the region of Madhya Pradesh. In India, there is an indigenously developed vaccine which is both

of prophylactic and therapeutic in nature. Hence, vaccination against paratuberculosis is recommended for the susceptible livestock population of the area in the state to contain economic losses to the livestock owners.

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Conflict of interest

All authors declare that they have no conflict of interest.

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