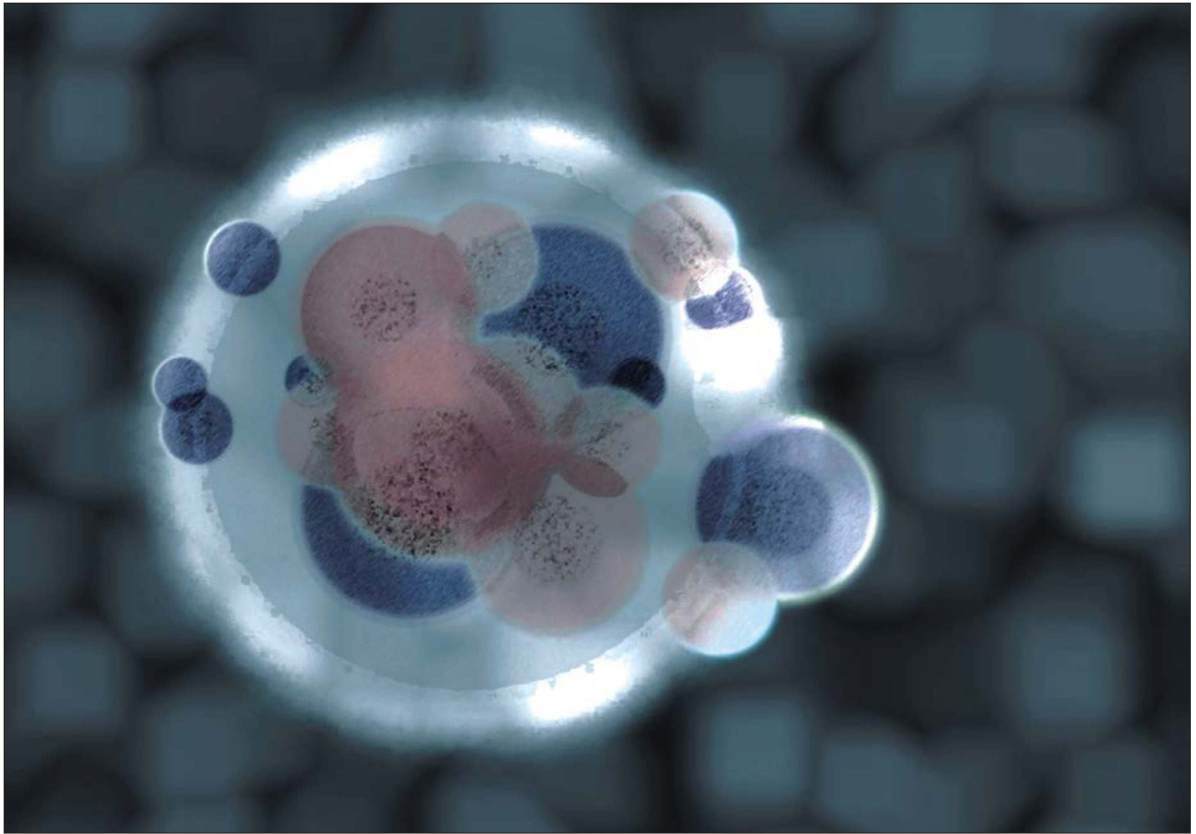

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Johne's Disease (JD) in a High Yielding Holstein Friesian Cattle Dairy Farm in India

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Abstract: Bovine Johne's Disease (BJD) is a chronic granulomatous enteritis that affects ruminants worldwide and is having significant impact on the world economy and has been frequently reported from farm and farmer's herds. An attack of Johne's disease in a newly established cattle dairy farm consisting of high yielding Holstein Friesian (HF) cows in the Alwar district of Rajasthan was investigated for the first time in India. Since slaughter of cows is prohibited in India therefore management of bovine JD is critical for the success of dairy industry in the country and in this aspect the research paper is significant. Out of a total of 35 fecal samples screened by microscopy, 24 (68.5%) were positive for *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Screening of 26 serum and 23 milk samples by 'Indigenous ELISA kit' employing semi-purified antigen of native strain ('S 5') of MAP, 24 (92.3%) and 14 (60.8%) were positive, respectively. Sensitivity of 'Indigenous serum ELISA' with reference to fecal microscopy and milk ELISA was 88.2 and 90.0%, respectively. Screening of blood samples of 14 cows, by specific PCR (IS*900*), 5 (35.7%) were positive. Genotyping of PCR positive HF crossbred cows using IS*1311* PCR-REA showed presence of highly pathogenic 'Indian Bison type' genotype. Comparison of 3 tests (milk ELISA, fecal microscopy and IS*900* PCR) with 'Indigenous serum ELISA' revealed substantial agreement between tests. Study also reported serious economic losses in terms of productivity (reduced quality and quantity of milk), reduced fertility and conception, decreased body weight and growth rate which left the farmer economy devastated due to attack of Johne's disease in high yielding cattle herd of HF crossbred cows.

Key words: Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis*, indian bison type, cattle, holstein friesian, ELISA, PCR

INTRODUCTION

Bovine Johne's disease (BJD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic granulomatous enteritis affecting ruminants worldwide. Johne's disease is having significant impact on the world economy and has been frequently reported from farm and farmer's herds (Goswami *et al*., 2000; Kumar *et al*., 2007; Shroff *et al*., 2013). The disease is endemic in the population of domestic and wild ruminants (Sharma *et al*., 2008; Mishra *et al*., 2009; Singh *et al*., 2010a), primates and in human beings (Chamberlin and Naser, 2006; Singh *et al*., 2011). However, reports of BJD outbreaks are either limited or non-existent. Though India is foremost in milk production (127.3 million tonnes) globally but low per animal productivity of 210.8 million cattle heads is the major concern of Indian dairy industry (FAO, 2012). Johne's disease is a production disease leading to heavy economic losses to farmers and >70% of US dairy cattle

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herds have been reported to be infected with MAP causing annual average loss of $200-250 million (Ott *et al*., 1999; Chi *et al*., 2002) and losses to the dairy industry may exceed $1.5 billion/year (Wells and Wagner, 2000). Limited reports on BJD in cattle are available in the country (Sivakumar *et al*., 2005).

Dairy development in India was started under British period while Defense department set up dairy farms to make sure the milk and butter available to army. Field scale cross breeding of non-descript cattle was initiated under aegis of 'Intensive Cattle Development Project (ICDP), Government of India in 1964 as policy to increase milk production. Large scale cross breeding programs were followed by Bharathiya Agro-Industries Foundation (BAIF) in 1970s. In 1974, National Commission on Agriculture (NCA) also endorsed cross breeding as a major strategy and potent instrument to boost milk production of native cattle. However, momentum and economic relevance to cross breeding was provided in mid seventies, with development of extensive dairy cooperative network under 'Operation Flood', which provided market stimulus and price support for milk. Though four foreign breeds introduced were Holstein Friesian, Red Dane, Jersey and Brown Swiss but crossbreeding with Holstein Friesian (HF) boosted distinctive markings and outstanding milk production of native cattle breeds. HF crosses have potential to yield good quantity of milk (3400-3600 kg milk year^{-1}) (Barbaruah and Joseph, 2008) and by 2003, the population of cross bred cattle was 24.686 million (13.3%) as compared to 160.495 million (86.6%) indigenous or native. However, high yielding HF dairy cattle suffered from different kinds of stresses, nutrition (quality and quantity of feed and fodder), environment (hot and humid weather) and diseases (blood protozoan, reproductive diseases, sub-clinical mastitis, *M. bovis* infection etc (Dohoo and Martin, 1984; Tadayon *et al*., 2008; Vahora *et al*., 2012; Mortier *et al*., 2013). But status of Johne's disease has never been investigated HF crosses.

Traditionally clinical animals (cattle and buffaloes) suffer from gradual loss in body condition, body weights and diarrhea, which once sets in is not treatable and animals continue in diarrhea (6 months to >3 years) till death. Animals also suffer from production losses (quantity and quality of milk), reduced fertility, decreased growth rate, increased incidence of mastitis etc. Besides shedding of MAP in feces viable MAP bacilli are present in the milk (Ayele *et al*., 2001; Grant *et al*., 2002) of clinical and sub-clinical animals. MAP has been recovered from pasteurized milk and milk products (Slana *et al*., 2008; Shankar *et al*., 2010) and environment (soil and water resources) (Singh *et al*., 2012). Since slaughter of cows is prohibited in India therefore management of bovine JD is very critical for the success dairy industry in the country. Present study investigated a rare attack of Bovine JD in the recently established dairy farm of high yielding cross bred HF cows located in the Alwar district of Rajasthan on the request of first information report (FIR) of local veterinary officer, who suspected that cows were suffering with BJD the basis of symptoms (loss of condition and sharp decline in productivity).

MATERIALS AND METHODS

Animals: A commercial cattle dairy farm was established in June 2011 in the Alwar district (10 km in east), Rajasthan by purchasing 20 Holstein Friesian (H/F) crossbred cows (10 adults and 10 calves) from farmer's herds. HF cross bred cows were purchased to increase the farm productivity in terms of milk. Present strength of cows in the farm was 71 [17 (0-18 months), 11 (18-30 months) and 43 (>30 months)]. Nine calves (2-11 months of age) died showing symptoms of weakness except one calf which had diarrhea. Clinically cows were unthrifty and poor in body conditions (suffered with problems of weight loss, weakness without diarrhea) was observed in the cows with reduced milk production in 2013. Fecal, blood, serum and milk samples of 35 cows (26 adult and 9 calves) were collected and screened for the presence *Mycobacterium avium* subspecies *paratuberculosis* using multiple diagnostic tests. Cows were inseminated by AI (100.0) by procuring semen of HF from semen bank at Bassi, Jaipur, Rajasthan.

Nutritional status and management of animals: The nutritional status of cows was good at the dairy farm. Cows were maintained on optimum nutrition under intensive management system (feeding on green fodder, conserved forage, crop residues and increased use of concentrates) with good hygienic conditions.

Fecal microscopy: Approximately, two gram of feces was thinly grounded in sterilized pestle and mortar with sterilized distilled water (10-12 mL) and centrifuged at 4000 rpm for 45 min at room temperature (RT). Supernatant was discarded, 2 smears were prepared from middle layer, stained by Ziehl Neelsen (ZN) and examined in oil immersion (100X) for Acid-fast Bacilli (AFB) indistinguishable to MAP. Nearly 100 fields were screened and samples were categorised as super, moderate and low shedders, respectively.

Indigenous serum ELISA: Serum samples (n = 26) were screened by 'Indigenous serum ELISA', developed for

goats and sheep by Singh *et al.* (2007) and was standardized for bovine as per Yadav *et al.* (2008). Absorbance readings were taken at 450 nm in an ELISA reader (iMark, BioRad). Suitable controls (positive and negative) were also kept while screening the serum samples. The S/P ratios were calculated from the Optical density (OD) values as per Collins (2002). Positive and strongly positive animal categories of S/P ratio were recorded as positive for MAP infection.

Milk samples: The 23 milk samples (12-15 mL) collected aseptically were centrifuged at 3000 rpm 45 min to split fat, whey and sediment layers. Whey (8 mL) was further reacted with 3.0% citric acid, clear whey collected after centrifugation for use in milk-ELISA and was stored at 4°C.

Indigenous milk ELISA: ELISA was carried out as per method of Sharma *et al.* (2008) with little modifications. Briefiy, blocking of antigen coated (0.1 μg of antigen well^{-1}) 96 wells ELISA plates was done by adding 100 μL of 3% skimmed milk in phosphate buffer saline (PBS) and incubation at 37°C for 45 min. Then the plate was washed three times with PBS Tween-20 (PBST). After washing, 100 μL of 1: 4 milk whey samples diluted in buffer (1% BSA in PBST) were poured to each well in duplicates and the plate incubated for 2 h at 37°C. Washing steps were again repeated with PBST. After this, reaction with 100 μL of 1:5000 rabbit anti-bovine horseradish peroxidase (HRPO) conjugate (Bangalore Genei, India) in PBS (pH 7.4) was followed and the plate incubated for 50 min at 37°C. The plate was washed thrice with PBST and 100 μL of fresh substrate [5 mg plate^{-1} in substrate buffer (pH, 5.0)] (OPD, Sigma) poured in to all the wells. The plate was finally incubated for 30 min in dark atmosphere at 37°C and the absorbance read at 450 nm in an ELISA reader (iMark, BioRad).

Molecular characterisation

IS*900* PCR: DNA was extracted from blood samples (n = 14) and subjected to specific IS*900* PCR following the protocol of Singh *et al.* (2010b) with little modifications. Briefly, in a volume of 12.5 μL of 2X red dye PCR master mix (Genei), 1 μL each of forward (10 pmol μL) and reverse primer (10 pmol μL), 7.5 μL of nuclease free water and 3 μL template DNA were added (25 μL total volume). Thermal cycling conditions used were: Initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 sec), annealing (64°C for 30 sec), extension (72°C for 30 sec) and a final extension (72°C for 10 min). Product size (413 bp) was considered positive, after separation on 1.8% agarose gel electrophoresis. MAP IS*900* primer sequences used as per Millar *et al.* (1996) were: P90 5'-GAAGGGTGTTCG GGGCCGTCGCTTAGG-3' (Forward primer) and P91 5'-G GGTTGAGGTCGATCGCCCACGTGAC-3' (Reverse primer).

IS*1311* PCR: IS*900* PCR positives were subjected to IS*1311* PCR using forward (M56) and reverse (M119) primers as per Sevilla *et al.* (2005). Briefly, in a volume of 15 μL of 2X red dye PCR master mix (Genei), 1 μL each of forward (10 pmol μL) and reverse primer (10 pmol μL), 10 μL of nuclease free water and 3 μL of template DNA was added (total volume 30 μL). Thermal cycling conditions were as follows: Initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 sec), annealing (62°C for 30 sec), extension (72°C for 1 min) and a final extension (72°C for 10 min). An amplicon size of 608 bp visualized after running the PCR products on 1.8% agarose gel stained with ethidium bromide was considered as positive for MAP.

IS*1311* PCR-restriction endonuclease analysis (REA): IS*1311* PCR-Restriction endonuclease analysis (PCR-REA) was carried out employing endonuclease enzymes as (*HinfI* and *MseI*) following the method of Sevilla *et al.* (2005) with modifications. Briefly, REA reaction was performed in a 30 μL volume containing 20 μL positive IS*1311* PCR product, 5 μL 10X reaction buffer and 1 μL (2U) each of *HinfI* and *MseI* endonucleases (Fermentas) and reaction mixture incubated at 37°C for 2 h. Digested products were visualized on 2% agorose gel electrophoresis and genotypes (band patterns) were interpreted as described by Whittington *et al.* (2001).

Statistical analysis: Proportional agreement between the tests and sensitivity of kit were compared as per Arizmendi and Grimes (1995).

RESULTS

Fecal microscopy: Of the 35 fecal samples screened by microscopy, 24 (68.5%) were positive for MAP infection and 4, 4 and 16 animals were +3, +2 and +1 level shedders, respectively (Table 1).

Indigenous ELISA kit: Screening of 26 serum and 23 milk samples by 'Indigenous serum ELISA' using antigen from native strain ('S 5') of MAP revealed 24 (92.3%) and 14 (60.8%) cows to be MAP positive, respectively (Table 1, 2). Sensitivity of 'Indigenous serum ELISA'

Table 1: Screening of HF cows for MAP infection by multiple tests

	Results			
Test	Microscopy	Serum ELISA	Milk ELISA	IS*900* blood PCR
Animals (n)	35	26	23	14
Positive n (%)	24 (68.5)	24 (92.3)	14 (60.8)	5 (35.7)

Figures in parentheses are percentage

Table 2: Comparative evaluation of S/P ratios of the results of serum and milk ELISA

S/P ratio	Disease status	Indigenous ELISA animals (n)	Milk ELISA animals (n)
0.00-0.09	Negative	Nil	Nil
0.10-0.24	Suspect	Nil	04 (17.3)
0.25-0.39	Low positive	02 (7.6)	05 (21.7)
0.40-0.99	Positive	23 (88.4)	14 (60.8)
1.0-10.0	Strong positive	01 (3.8)	Nil
Total animals	26	23	

*Positive and strong positive in S/P ratio taken as positive, Figures in parentheses are percentage

Table 3: Comparative evaluation of Indigenous ELISA and milk ELISA

Indigenous serum ELISA	Milk ELISA Positive (%)	Negative (%)	Sensitivity (%)
Positive	10/17 (58.8)	6/17 (35.2)	100.0
Negative	0/17 (0.0)	1/17 (5.8)	

Figures in parentheses are percentage, Agreement between Indigenous ELISA kit and Milk ELISA-64.7%

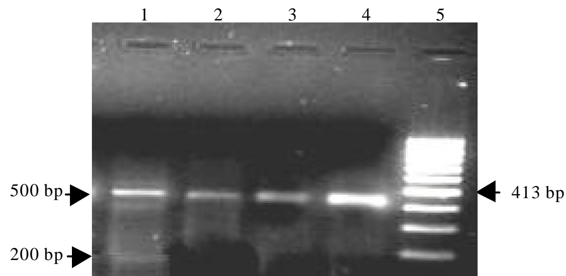

Fig. 1: MAP specific amplicons (413 bp) using IS*900* blood PCR, Lane 1: 100 bp ladder, Lane 2: Positive control (MAP DNA), Lane 3-5: DNA samples isolated from blood

compared to fecal microscopy and milk ELISA was 88.2 and 100.0%, respectively (Table 3 and 4).

IS*900* PCR and genotyping: Of the 14 HF crossbred cows screened, 5 (35.7%) were positive in IS*900* blood PCR (Fig. 1 and Table 1). All the IS*900* PCR positive samples yielded positive reaction in IS*1311* PCR testing. Bio-typing of 5 positive samples in IS*1311* PCR showed presence of highly pathogenic 'Indian Bison type' restriction pattern in HF cross bred cattle dairy farm using IS*1311* PCR-REA (Fig. 2 and 3).

Table 4: Comparative evaluation of indigenous ELISA and fecal microscopy

	Combinations			
Tests	1	2	3	4
Indigenous ELISA	+	-	+	-
Microscopy	+	-	-	+
Total (22)	15 (68.1)	1 (4.5)	4 (18.1)	2 (9.0)

Figures in parentheses are percentage. Agreement between indigenous ELISA kit and microscopy-72.7%, sensitivity-88.2%

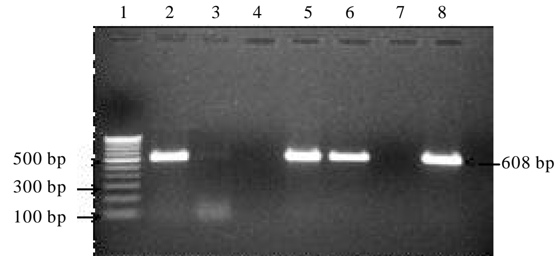

Fig. 2: MAP specific amplicons (608 bp) using IS*1311* blood PCR, Lane 1: 100 bp DNA ladder, Lane 2: Positive control (MAP DNA), Lane 4: Negative control (miliQ water) and Lane 5-8: DNA samples positive in IS*900* PCR

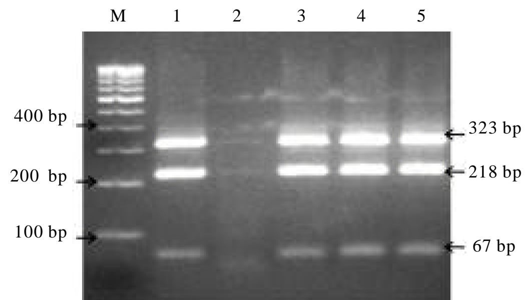

Fig. 3: IS*1311* PCR-REA analysis, Lane M: 100bp DNA ladder, Lane 1: Positive control (MAP Indian Bison type DNA), Lane 2: Negative control (miliQ water), Lane 3-5: Digested DNA sample

DISCUSSION

Population trends of Indian cattle showed a decline of 9% in cattle population during 1992 to 2003, mostly confined to indigenous cattle stocks of 87% in 2003. Government of India used up decades on encouraging cross-breeding of native dairy cattle with foreign bull breeds like Holstein. Therefore the indigenous cattle population declined by 15.0% while the cross-breeds amplified by 62.0%. This decline was specifically drastic for males (62.0%) among the indigenous stock. The shift in farmer's preference was mainly driven by the

high productivity of HF cross bred cow. Though cross-breeding program in Indian dairy cattle boosted milk production at higher rates but is not appropriate for smallholder and landless farmers, who own 68.0% of India's dairy cattle. Cross breeding program was not as successful as envisaged. Primarily HF crossbred cattle required expensive shelters, which small farmers cannot afford. Secondly, India's loss of cattle breed diversity mirrors potentially dangerous global trends, including in the US, where 93% of dairy cattle are now Holsteins. Thirdly high yielding HF and HF crosses suffered required high quality nutrition and with many types of health problems including blood protozoan infection, reproductive diseases, sub-clinical mastitis, *M. bovis* infection etc., (Dohoo and Martin, 1984; Tadayon *et al*., 2008; Vahora *et al*., 2012), however, status of Johne's disease has never been investigated. Due to ban on cow slaughter the number of low and unproductive cows has increased in the country. Prevalence of MAP thus may be significantly higher in cattle as compared to goats, sheep and buffaloes.

Due to poor sensitivity and specificity of Johnin, at present there is no genuine field-based test. For diagnosis of Johne's disease range of diagnostics e.g., Johnin, fecal microscopy, fecal culture, PCR (fecal and blood) and antibody assays (ELISA and IFN-α) are available. However, no single test could precisely diagnose all cases with complete accuracy (Tiwari *et al*., 2006). Therefore, multiple tests were used in the present study to investigate the attack of suspected Johne's disease in the HF cross bred dairy farm in Alwar district of Rajasthan. Comparative evaluation of fecal microscopy with 'Indigenous serum ELISA' showed that 68.5% cows were positive by fecal microscopy as compared to 'Indigenous serum ELISA' (92.3%). Proportional agreement between 'Indigenous ELISA' and fecal microscopy was substantial (72.7%), making sensitivity 88.2% with 11.7% false negative results. High sero-prevalence of BJD in HF crossbred cows correlated with poor per animal productivity and other economic losses. Using 'Indigenous ELISA kit', Singh *et al.* (2008) reported moderate prevalence (32.9 and 25.0%) of BJD in Uttar Pradesh and Punjab region of the country, respectively. Employing different antigens, variable sensitivity of serum ELISA (45.7-70.0%) was recorded (Vannuffel *et al*., 1994; Collins *et al*., 2005). Though ELISA is fast, affordable and widely used test however sensitivity of many commercial ELISA kits available in the market is very low when used in Indian conditions (Singh *et al*., 2009).

Milk ELISA standardized employing indigenous protoplasmic antigen of the native strain of MAP 'S 5' of goat origin detected 60.8% (14/23) lactating HF crossbred cows positive for MAP lacto-antibodies. However, proportional agreement between 'Indigenous serum ELISA' and milk ELISA was 64.7%, making sensitivity 100.0% with no false negative results. Muskens *et al.* (2000) reported variable MAP prevalence in different countries by employing ELISA (serum and milk). Sweeney *et al.* (1994) reported 50.0% sensitivity in cattle milk using LAM ELISA. In the present study, indigenous protoplasmic antigen from native strain of MAP 'S 5' was sensitive at 0.1 μg of antigen per well concentration with 1:4 whey dilution. However, Salgado *et al.* (2005) screened MAP lacto-antibodies at 1:2 dilutions of whey. Study showed a high correlation between animals positive in milk ELISA and 'Indigenous serum ELISA'. Based upon the above findings milk ELISA using antigen from native strain of MAP of goat origin has the able potential to be used as a 'Herd screening test' for cows in lactation.

Of the 14 HF crossbred cows screened, 5 (35.7%) were positive in IS*900* blood PCR as confirmatory test. Presence of MAP in IS*900* blood PCR indicated that MAP gets circulated to other organs than intestinal tract (Hines *et al*., 1987; Sohal *et al*., 2009, 2010) through blood cells. secondly, MAP being intracellular orgamism is disseminated by blood phagocytes to extra intestinal sites (Zurbrick and Czuprynski, 1987; Windsor and Whittington, 2010; Singh *et al*., 2013a). As expected, all IS*900* PCR positive cows were positive in the IS*1311* PCR and showed the prevalence of highly pathogenic MAP 'Indian Bison type' restriction pattern in HF cross bred cattle dairy farm using IS*1311* PCR-REA. Proportional agreement between 'Indigenous serum ELISA' and IS*900* blood PCR was 60.0%, making sensitivity 100.0% with no false negative results (Table 5). However, proportional agreement between IS*900* blood PCR and fecal microscopy was 78.5%, making sensitivity 62.5% (Table 6).

Table 5: Comparative evaluation of indigenous ELISA and IS*900* PCR

	IS*900* PCR		
Indigenous ELISA	Positive (%)	Negative (%)	Sensitivity (%)
Positive	4/10 (40.0)	4/10 (40.0)	100
Negative	0/10 (0.0)	2/10 (20.0)	

Figures in parentheses are percentage. Agreement between indigenous ELISA and IS*900* PCR-60.0%

Table 6: Marison of IS*900* PCR and fecal microscopy for presence of MAP infection

		Microscopy positive			
Tests	Microscopy egative	1+	2+	3+	Total
IS*900* PCR positive	0	2	1	2	5
IS*900* PCR negative	6	3	0	0	9
Total	6	5	1	2	14

Agreement between Indigenous ELISA and fecal microscopy-78.5%. Sensitivity-62.5%

High prevalence of JD has been recorded by other workers in goats (Kumar *et al*., 2007), sheep (Singh *et al*., 2013b), cattle and buffaloes (Singh *et al*., 2008) and from many farm herds located in the North India (Goswami *et al*., 2000; Kumar *et al*., 2007). Srivastav and More (1987) reported low prevalence due to use of less sensitive tests (Johnin and fecal microscopy) as compared to ELISA used in this study. Lowered sensitivity as compared to present findings may be due to poor standardization of the procedure.

The advances in molecular diagnostic tools (Deb *et al*., 2011, 2013; Dhama *et al*., 2013a, 2014), efficacious vaccines (Dhama *et al*., 2008, 2011, 2013b) and emerging therapeutic modalities (Mahima *et al*., 2012; Dhama *et al*., 2013c, 2013d; Tiwari *et al*., 2013a) need to be explored for effective prevention and control of MAP infection (Johne's disease), an economically important pathogen having public health concerns (Tripathi *et al*., 2002; Grant, 2005; Sweeney *et al*., 2012). This is particularly important in the present One Health, One Medicine concept and emerging scenario drug resistance in bacterial pathogens (Hermon-Taylor, 2009; Momotani, 2012; Dhama *et al*., 2013e; Tiwari *et al*., 2013b).

In conclusion, estimation of the bio-load of Johne's disease (JD) at Holstein Friesian cattle dairy farm revealed higher prevalence of JD in this region, which indirectly reflects economic losses being suffered by the dairy industry in the country. Appropriate biosecurity measures need to be practiced at these cattle dairy farms with a check on the wandering of such animals. This can be done by controlling the use of their dung in the close by agro-farms and grasslands which could pose a continuous source of exposure to MAP infection for the susceptible domestic animal population and especially in cross-breda cows.

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