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ORIGINAL ARTICLE

'Bio-Load' and Bio-Type Profiles of *Mycobacterium avium* Subspecies *paratuberculosis* Infection in the Domestic Livestock Population Endemic for Johne's Disease: A Survey of 28 years (1985–2013) in India

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Summary

Bio-load and bio-profile of *Mycobacterium avium* subspecies *paratuberculosis* was studied in the domestic livestock population of the country. Of the 23 429 farm and farmer's animals screened, average bio-load was 23.3% (Period of study; 28 years for goats; 13 years for sheep, cattle and buffaloes). Species-wise, bio-load was 20.1, 32.7, 39.3 and 28.3% in goats, sheep, cattle and buffaloes, respectively. Bio-load was significantly lower in time period A ($P < 0.001$) and B ($P < 0.03$), compared with period C. Geographical zone-wise, bio-load of MAP was significantly higher ($P < 0.05$) in Central zone compared with South, West, East and North zones. Bio-load in 11 states ranged from 16.2 to 87.8%. Of 8450, 5643, 8185 and 1151 samples screened by microscopy, culture, indigenous ELISA and IS*900* blood PCR, 20.0, 10.6, 35.1 and 26.6% samples were positive, respectively. Bio-load was 32.8 and 31.6% in farm and farmer's goats and sheep, respectively, and 62.1% in farmer's cattle. MAP bio-load was also monitored in four farm units (three goats and one sheep) for breed improvement and three farm goats units for experimental purposes at Central Institute for Research on Goats in Mathura district. Of the 8025 goats and 1525 sheep that died from 1988 to 2013, 10.9 and 3.0% deaths were due to JD, respectively. On the basis of JD and suspected JD, 10.0 and 28.4% goats and 2.2 and 40.9% sheep, respectively were culled from the farm units in 25 years. On microscopy of 214 tissues (mesenteric lymph nodes and intestines) of 107 animals, it was observed that bio-load was high (25.0–60.0%) in farm animals. 'Indian Bison Type' was dominants biotype, irrespective of domestic livestock species and the geographical zone.

Introduction

Johne's disease (JD) is a progressive, incurable, chronic, debilitating and wasting disease of domestic ruminants, characterized by weight loss and chronic diarrhoea. *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the cause of JD, has the widest host range and inter-species sharing of MAP and has been frequently reported (Sevilla et al., 2005). In endemic herds, only few animals may develop clinical signs, whereas majority remain asymptomatic (subclinical) shedders. Though mortality rates are low, >50% animals in a herd may be

asymptomatic shedders. Low per-animal productivity is the characteristic of native breeds of domestic ruminants. JD is the most important infection of domestic ruminants affecting per-animal productivity world-wide. In USA, >70.0% of dairy and 8.0% beef herds had MAP infection (Gonzalez-Juarrero et al., 2012). In India, except isolated reports, there is no systematic study on the monitoring of MAP infection in animals and losses caused to the animal production system at the national level. In the absence of monitoring and control measures, bio-load of JD has increased steadily in domestic livestock population of the country. The present study reports bio-load of MAP in the domestic livestock population (farm and farmer's herds and flocks) based on samples (faeces, tissues, serum, milk and blood) submitted and screened for Johne's disease in the last 28 years (1985–2013) using microscopy, culture, ELISA and PCR tests.

Ethical Approval

Work has been approved by the Ethical Committee of the institute (registration no., 207 with Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt., of India).

Materials and Methods

Data presented in this study is not based on random sampling of the domestic livestock species; therefore, the term 'Prevalence' was not used. As the information is based on screening of animals suspected of suffering from JD in naturally infected farm and farmer's herds and flocks, therefore, to represent the probable level of disease in animals, the term 'Bio-load' was used. Similarly, Nielsen and Toft (2009), in a review of prevalences of MAP in farm animals in Europe based on a wide range of studies, concluded that prevalences of MAP would have to be guess-estimates based on available data, as comparable true prevalences estimates could rarely be calculated. Major critical issues identified in majority of studies and as recorded in the present study were primarily due to lack of knowledge of the test accuracy of diagnostic test used or due to studies where study population did not reflect the target population. Samples submitted were driven from domestic livestock species (goats, sheep, cattle and buffaloes) belonging to farm and farmer's herds and flocks. Initially (1985–2000) samples were driven primarily from goats population located at CIRG, Mathura and from other farms and farmer's herds. In 28 years of monitoring of MAP infection, we studied different aspects of JD, primarily to sustain and conserve the herds of three important native goat breeds (Jamunapari, Jakhrana and Barbari), a flock of Muzaffarnagri sheep and three other experimental goatherds located at the CIRG, Mathura. Facility for the diagnosis of MAP infection was also extended to other farm and farmer's herds and flocks in the country. Native strains of MAP from goats were bio-typed as similar to 'Bison Type' by Dr R. J. Whittington, (Elizabeth Mac Arthur Agricultural Institute, Australia) using IS*1311* PCR-REA (Whittington et al., 2001). This report was instrumental in expanding the study to sheep, cattle and buffaloes, to know the biotype of MAP. Bio-typing of MAP isolates from sheep, cattle and buffaloes were also found to be similar to 'Bison Type' by Dr. R.A. Juste laboratory in Spain in 2004. Therefore, the period of study (28 years) has been broadly divided into three time zones. To understand the changes in bio-load and bio-type profile of MAP, with respect to time period, domestic livestock species, geographical locations and diagnostic tests, three times zones were further subdivided in to '5 year' time intervals.

Animals and samples

Low body weights, late maturity, increased kidding interval, high and early culling and low productivity are the hallmarks of native livestock breeds. Samples from domestic livestock population were driven from animals belonging to 14 states [Uttar Pradesh (UP), Himachal Pradesh (HP), Punjab and Haryana in the North, Odisha, West Bengal and Assam in the East, Gujarat, Rajasthan and Maharashtra in the West, Madhya Pradesh (MP) in the Central zone and Kerala, Tamil Nadu (TN) and Karnataka in the South] of the country over the last 13 years (2000–2013). Animals screened belonged to both farm and farmer's herds and flocks, wherein semi-intensive and extensive management systems were practiced, respectively. As JD is endemic in domestic ruminants, samples submitted from other parts of the country were suspected to belong to animals suffering from subclinical or clinical JD (weakness, diarrhoea, poor body condition).

Bio-load of MAP was also monitored in the farm animals (six goatherds and one sheep flock) reared under semi-intensive management system at CIRG, Mathura for genetic improvement through selective breeding, in the last 28 years (1985–2013). The institute maintains nucleus herds of nearly 2500 goats and 700 sheep for the conservation, multiplication and distribution of the elite germ plasm to farmers. Farms of Barbari and Jamunapari goats and Muzaffarnagri sheep were established in 1979, while the Jakhrana goat breed was introduced in 2005. Besides goat-breeding farms, three experimental goat units [unit 1 for nutritional trials, unit 2 for physiology and reproduction studies and unit 3 for health studies] are also maintained. These six farm units are naturally infected and endemic for JD and were therefore regularly monitored for

MAP infection as an important health management activity. During daily treatment of goats and sheep at the farms, if any animal exhibited signs of weakness, repeated diarrhoea, loss in body weights, stunting, emaciation, not responding to treatment etc., was suspected for JD. The suspected animals were regularly screened for MAP infection by microscopy, and those found positive in microscopy were culled from the farms. Test and cull policy for the control of JD has continued at CIRG farm units since the establishment of institute and farm units as part of JD management and control strategy. Animals negative for MAP infection in faecal microscopy but suffering from weakness, un-thriftiness etc., were also culled from the livestock farms on health grounds. Therefore, information on culling was also collected between 2011 and 2013 and analyzed. Tissues (MLN and intestines near the ileo-caecal junction) collected at necropsy from the animals that died naturally or slaughtered for meat production were also screened against MAP infection by impression smear microscopy. Faeces and serum samples were the most common samples submitted. However, other samples like blood, milk and tissues were also submitted for screening. A total of 23 429 samples of goats, cattle, sheep and buffaloes were screened in the past 28 years (1985–2013).

Time period (1985–2013)

To present 'bio-load' of MAP in 28 year period, the data was pooled for five-yearly intervals to demonstrate change in 'percent bio-load' of MAP from 1985 to 2013. As JD is a chronic infection, the changes if any are expected to be slow. Year 2000 was used as the cut off year when for the first time the 'bio-type' profile of MAP strains infecting goats was identified as 'Bison type'. Therefore, after 2000, other livestock species (sheep, cattle & buffaloes) were also included in screening against JD to know the 'percent bio-load' and bio-type profile of MAP. The 28 years time period was further subdivided as before (1985–2000) and after 2000 (2001–2010) and as the remaining period (2011–2013) was less than 5 years, it was therefore kept separately (Time period C).

Time zone A (1985–2000): The first fifteen-year time zone consisted of 3 five-yearly intervals (1985–1990, 1991–1995 and 1996–2000), wherein 10 252 samples of goats were screened by microscopy, culture and ELISA (Tables 1, 2 and 3).

Time zone B (2001–2010): The second ten-year time zone consisted of 2 five-yearly intervals (2001–2005 and 2006–2010), wherein 7099 animals of four domestic livestock species and were screened using microscopy, culture, ELISA and PCR (Tables 1, 2 and 3).

Time zone C (2011–2013): The third 3-year time zone consisted of a 3-year interval (2011–2013), wherein 6078 animals of four domestic livestock species were screened using microscopy, PCR and ELISA (Tables 1, 2 and 3).

Screening of samples

Samples were screened using multiple tests [Microscopy, culture, IS*900* PCR (faecal, tissues, milk and blood) and indigenous ELISA tests (serum and milk)].

Faecal samples

Faecal samples were concentrated by centrifugation and stained with Ziehl-Neelsen (ZN) stain. Pink coloured acid-fast short bacilli indistinguishable to MAP were considered positive for MAP infection. Samples were cultured as per Singh et al. (1996) for the isolation of MAP. Briefly, samples were centrifuged, decontaminated

Table 1. Livestock species and time period-wise percent bio-load of MAP in domestic livestock species (1985–2013), a significance of the difference in bio-load of MAP between three time period A, B and C and four animal species (goat, sheep, cattle and buffaloes)

Species	Period A			Period B		Period C[a]	Cumulative
	1985–1990	1991–1995	1996–2000	2001–2005	2006–2010	2011–2013	(1985–2013)
Goats	11.4 (464/4057)	13.1 (292/2221)	11.1 (443/3974)	21.5 (533/2479)	29.7 (443/1490)	35.1 (1640/4672)	20.1 (3815/18 893)
Sheep*	ND	ND	ND	25.0 (5/20)	25.9(118/454)	71.9(59/82)	32.7(182/556)
Subtotal	11.4(464/4057)	13.1 (292/2221)	11.1 (443/3974)	21.52 (538/2499)	28.8 (561/1944)	35.7 (1699/4754)	20.5 (3997/19 449)
Cattle*	ND	ND	ND	47.0(80/170)	31.0 (558/1799)	50.3 (634/1260)	39.3 (1272/3229)
Buffaloes*	ND	ND	ND	41.9(70/167)	22.1 (115/520)	43.7(28/64)	28.3 (213/751)
Subtotal	11.4(464/4057)	13.1 (292/2221)	11.1 (443/3974)	44.5 (150/337)	29.0 (673/2319)	50.0 (662/1324)	37.3 (1485/3980)
Grand Total	11.4 (464/4057) 11.6 (1199/10 252)	13.1 (292/2221)	11.1 (443/3974)	24.2 (688/2836) 27.0 (1922/7099)	28.9 (1234/4263)	38.8 (2361/6078)	23.3 (5482/23 429)

ND, not done.

Figures in parenthesis are numbers (Positive/Total).

[a]Up to July 2013.

*$P < 0.05$ statistically significant.

**$P<0.001$ statistically significant; A–B $P < 0.03$, B–C $P < 0.03$ and A–C $P < 0.001$.

Table 2. Geographical region and period-wise percent bio-load of MAP in domestic livestock population (1985–2013)

Zone	States	Period A (1985–2000)	Period B (2001–2010)	Period C (2011–2013a)	Total (State-wise)
North*	1. UPb[&d]	11.5 (1167/10 130)[d]	27.7 (1131/4078)[b]	51.3 (851/1657)[b]	19.8 (3149/15 865)
	2. HP[c]	0.0 (0/14)	95.0 (19/20)	71.9 (59/82)	67.2 (78/116)
	3. Punjab[e]	ND	21.6(277/1277)	84.4 (76/90)	25.8 (353/1367)
	4. Haryana[e]	ND	19.2(22/114)	75.0 (9/12)	24.6 (31/126)
	Subtotal				20.6 (3411/17 474)
East*	5. Odisha[d]	33.7 (28/83)	ND	ND	33.7 (28/83)
	6. West Bengal[d]	16.0 (4/25)	ND	ND	16.0(4/25)
	7. Assam[d]	ND	ND	33.3 (63/189)	33.3 (63/189)
	Subtotal				31.9 (95/297)
West*	8. Gujarat[f]	ND	33.8 (118/349)	54.5 (368/674)	47.5 (486/1023)
	9. Rajasthan[d]	ND	26.4 (178/672)	56.1 (332/591)	40.3 (510/1263)
	10. Maharashtra[g]	ND	ND	16.2 (356/2187)	16.2 (356/2187)
	Subtotal				30.2 (1352/4473)
Central*	11. MP[c]	ND	85.7(12/14)	47.6(82/172)	50.5 (94/186)
	Subtotal				50.5 (94/186)
South*	12. Kerala[d]	ND	ND	87.8 (36/41)	87.8 (36/41)
	13. Tamil Nadu[d]	ND	28.6 (165/575)	29.4 (86/292)	28.9 (251/867)
	14. Karnataka[f]	ND	ND	47.2 (43/91)	47.2 (43/91)
	Subtotal				33.0 (330/999)
Total		11.6 (1199/10 252)	27.0 (1922/7099)	38.8 (2361/6078)	23.3 (5482/23 429)

ND, not done; UP, Uttar Pradesh, HP, Himachal Pradesh, MP, Madhya Pradesh.
Figures in parenthesis are numbers (Positive/Total).
[a]Up to July 2013.
[b]Goats, cattle, sheep, buffaloes.
[c]Goats & cattle.
[d]Goats.
[e]Cattle & buffaloes.
[f]Goats, sheep & cattle.
[g]Goats & sheep.
*$P < 0.05$ statistically significant.

Table 3. Diagnostic test-wise percent bio-load of MAP in domestic livestock species (1985–2013)

Species	Period	Diagnostic tests				Total
		Microscopy	Culture	ELISA	PCR	
Goats	1985–1990	9.1 (330/3614)	21.9 (64/291)	46.0 (70/152)	ND	11.4 (464/4057)
	1991–1995	ND	13.1 (292/2221)	ND	ND	13.1 (292/2221)
	1996–2000	17.3 (233/1346)	6.8 (138/2001)	11.4 (72/627)	ND	11.1 (443/3974)
Subtotal		11.3 (563/4960)	10.9 (494/4513)	18.2 (142/779)	ND	11.6 (1199/10 252)
Goat, Sheep, Cattle & Buffaloes	2001–2005	23.8 (245/1026)	8.8 (90/1018)	47.1 (236/501)	40.2 (117/291)	24.2 (688/2836)
	2006–2010	67.5 (175/259)	16.9 (19/112)	26.4 (966/3650)	30.5 (74/242)	28.9 (1234/4263)
Subtotal		32.6 (420/1285)	9.6 (109/1130)	28.9 (1202/4151)	35.8 (191/533)	27.0 (1922/7099)
Goat, Sheep, Cattle & Buffaloes	2011–2013 Subtotal	32.4 (715/2205)	ND	47.0 (1530/3255)	18.7 (116/618)	38.8 (2361/6078)
Grand Total		20.0 (1698/8450)	10.6 (603/5643)	35.1 (2874/8185)	26.6 (307/1151)	23.3 (5482/23 429)

ND, not done.
Figures in parenthesis are numbers (Positive/Total).
[a]Up to July 2013. 5

with hexadecyl pyridinium chloride at room temperature for 18–24 h and cultured on Herrold's egg yolk medium (HEYM) with and without mycobactin J. Slants were examined for appearance of colonies at weekly intervals up to 120 days. MAP colonies growing only on HEYM with mycobactin J were identified by slow growth,

mycobactin J dependence, acid-fastness, colonial and bacterial morphology.

Tissue samples

Smears made from tissues (MLN & intestines near ileo-caecal junction) from necropsied and slaughtered animals were screened for the presence of MAP. Smears were stained with ZN stain. Method standardized for culture of faecal samples (Singh et al., 1996) was used for culture of tissues.

DNA isolation and IS*900* PCR

DNA was isolated as per the method of van Soolingen et al. (1993). DNA was amplified by PCR using MAP specific IS*900* primers (Vary et al., 1990). The 229-bp fragment targeting specific IS*900* sequence was amplified from template DNA.

Is*1311* pcr

IS*900* PCR positives were subjected to IS*1311* PCR using M*56* and M119 primers as per Sevilla et al. (2005) with some modifications. Briefly, PCR was set up in volume of 25 μl, using 0.5–1.0 ng template DNA and 12.5 μl of 2X master mix (Promega, Madison, WI, USA). Thermal cycling was as follows: initial denaturation at 94°C for 3 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. An amplicon size of 608 bp was considered positive in IS*1311* PCR after separation on a 2% agarose gel stained with ethidium bromide.

Is1311 pcr_rea

IS*1311* PCR_REA was carried out as per Sevilla et al. (2005). Briefly, the reaction was carried out in a volume of 30 μl, containing 20 μl positive IS*1311* PCR product, 3 μl 10X buffer and 2 U of each endonuclease *Hinf*I and *Mse*I (Fermentas, USA). The reaction mixture was incubated at 37°C for 2.0 h. Band patterns were visualized after electrophoresis on a 4% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted as per Whittington et al. (2001)

Screening of serum by 'Indigenous ELISA kit'

The 'Indigenous ELISA kit' developed for screening of goats and sheep, at CIRG, Mathura, using protoplasmic antigens (PPA) from native Indian 'Bison type' biotype (Sohal et al., 2009; Singh et al., 2013a) of goat origin was employed for screening of serum samples. Positive and negative serum controls were selected from goats and cattle, positive and negative in culture in earlier studies. OD values were transformed to S/P ratios to estimate status of JD (Collins, 2002). Animals in strong positive category were considered positive.

Statistical analysis

To compare the bio-load of MAP within species, time period and geographical zone-wise, chi-square test was performed and analyzed using GRAPH PAD PRISM 5. Value of $P < 0.05$ were considered statistically significant.

Results

The study reports cumulative bio-load of MAP on the basis of samples (faeces, tissues, serum, milk and blood) submitted to Animal Health Division of Central Institute for Research on Goats (CIRG), Mathura (Uttar Pradesh, India) over the last 28 years (1985–2013).

Farm and farmer's animals from different regions of country

Domestic livestock species, time period, geographic region, diagnostic tests, management system and breed-wise bio-load of MAP in 28 years (1985–2013)

Of the 23 429 clinical (faecal, serum, blood, milk) and necropsy/slaughter house (tissues and serum) samples from goats, sheep, cattle and buffaloes screened by multiple tests (microscopy, culture, ELISA and PCR) over the last 28 years (1985–2013), the cumulative average bio-load of MAP was 23.3% (Table 1). Livestock species-wise average bio-load in goats, sheep, cattle and buffaloes was 20.1, 32.7, 39.3 and 28.3%, respectively (Table 1). Bio-load was significantly lower in goats ($P < 0.05$), compared with sheep, buffaloes and cows. The average bio-load of MAP in 28 years (1985–2013) time period showed an increasing trend and was 11.4, 13.1, 11.1, 24.2, 28.9 and 38.8% at five-yearly intervals of 1985–1990, 1991–1995, 1996–2000, 2001–2005, 2006–2010 and 2011–2013, respectively (Table 1). Time zone-wise, the average bio-load was 11.6, 27.0 and 38.8%, in 15- (1985–2000), 10- (2001–2010) and 3- (2011–2013) year periods, respectively. Which indicated significantly increasing trend between time zone A and B ($P < 0.03$), between B and C ($P < 0.03$) and between A and C ($P < 0.001$). Of the 11 states of country from where samples were submitted and analyzed, the average bio-load of MAP was 19.8, 67.2, 25.8, 24.6, 33.7, 16.0, 33.3, 47.5, 40.3, 16.2, 50.5, 87.8, 28.9, and 47.2% in the states of Uttar Pradesh, Himachal Pradesh, Punjab, Haryana, Odisha, West Bengal, Assam, Gujarat, Rajasthan, Maharashtra, Madhya Pradesh, Kerala, Tamil Nadu and Karnataka,

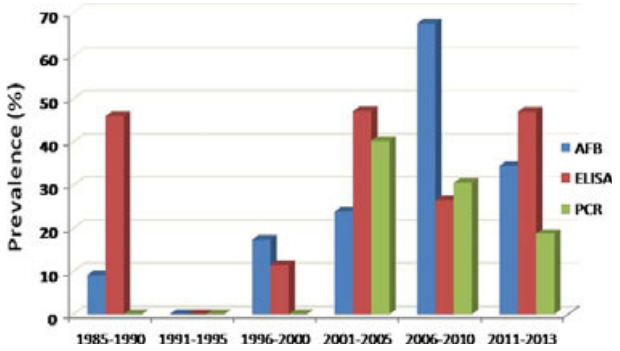

Fig. 1. Diagnostic test and time period-wise percent bio-load of MAP 12 in the domestic livestock (1985–2013).

respectively (Table 2). Geographical zone-wise, bio-load of MAP was 20.6, 31.9, 30.2, 50.7 and 33.0% in animals from North, East, West, Central and South zones, respectively (Table 2). Of the total 8450, 5643, 8185 and 1151 samples screened by microscopy, culture, indigenous ELISA and blood IS*900* PCR, 20.0, 10.6, 35.1 and 26.6% were positive, respectively (Table 3 and Fig. 1). Management system-wise, bio-load was 31.6 and 62.1% by screening of 240 goats and 175 cattle from farmer's herd (extensive management) using microscopy, ELISA and PCR. Whereas, in semi-intensive management system (farm herds and flocks of CIRG), bio-load was 32.8% by screening of 204 goats and sheep using three tests (Table 4).

Bio-load of MAP in farm goatherds and a sheep flock of CIRG, Mathura in 25 years (1988–2013)

On the basis of necropsy (mortality)

Of the total 8025 animals (6500 goats and 1525 sheep) that died and necropsied at CIRG, Mathura in the last 25 years (1988–2013), it was observed that 9.4% deaths were due to JD. Livestock species-wise, deaths due to JD were 10.9 and 2.9% in goats and sheep, respectively. Breed-wise, deaths due to JD were 6.7, 10.8 and 23.5% in Barbari, Jamunapari and Jakhrana breeds of native goats, respectively. Deaths due to JD were 17.4, 8.4 and 20.2% in the experimental goat units 1, 2 and 3, respectively. Of the 1525 deaths in Muzaffarnagri sheep unit, 2.9% died due to JD. Of the total 764 (9.5%) deaths due to suspected JD (weakness, stunting and unthrifty etc.), 8.3, 11.1, 29.2, 12.4, 7.4, 4.0 and 9.4% were from Barbari, Jamunapari, Jakhrana, Experimental unit 1, 2, 3 and Muzaffarnagri sheep, respectively (Table 5).

Table 4. Management system-wise percent bio-load of MAP in farm and farmer's animals using multiple tests (2011–2013)

Management system	Farm animals (semi-intensive)	Farmer's animals (extensive)	
Animal type/ number sampled	Goats & sheep/ (204)	Goats & sheep/ (240)	Cattle/(175)
Microscopy	42.7 (65/152)	29.2 (59/202)	52.0 (38/73
Blood PCR	12.9 (10/77)	3.1 (2/64)	40.0 (6/15)
Indigenous ELISA kit	33.0 (41/124)	47.2 (70/148)	70.2 (92/131)
Total	32.8 (116/353)	31.6 (131/414)	62.1(136/219)

Figures in parentheses are numbers (Positive/Total).
Microscopy –162/427 = 37.9%, Blood PCR –18/156 = 11.5%, ELISA kits –203/403 = 50.0%.

On the basis of screening of suspected goats and sheep (2011–2013)

A total of 234 goats and sheep were culled from CIRG farm units between 2011 and 2013. Of these, 8.5, 30.7 and 60.6% animals were culled on the grounds of infection with JD, suspected JD (weakness, stunting and unthrifty) and other diseases, respectively. Of 20 animals culled on the basis of JD, 9.5, 0.0, 25.0, 7.6, 7.1 and 2.2% animals belonged to Barbari, Jamunapari, Jakhrana, experimental unit 1 and 2 goatherds and in Muzaffarnagri sheep unit, respectively (Table 6). Animals suspected for JD and found negative in faecal microscopy (30.7%) were also culled on the basis of weakness, stunting and un-thriftiness, Total animal losses due to JD and suspected JD in the 3-year time period (2011–2013) were 26.1, 32.6, 47.7, 76.9, 35.7 and 43.1% in Barbari, Jamunapari, Jakhrana, experimental unit 1 and 2 goatherds and in Muzaffarnagri sheep unit, respectively (Table 6).

Screening of tissues of farm goats and sheep at slaughter and necropsy (2011–2013)

Screening of tissues of 82 apparently healthy and young (9 months) goats and sheep (slaughtered for meat production) by microscopy, 31.7 and 32.9% of mesenteric lymph nodes (MLN) and intestines were found positive, respectively. Screening of tissues of goats and sheep that died due to JD in this period, 40.0 and 60.0% of MLN and intestines were positive, respectively. Of the 20 goats and sheep that died due to causes (post-mortem diagnosis) other than JD, 25.0 and 30.0% of MLN and intestines of these animals were positive for MAP infection (Table 7).

Bio-type profile of MAP infecting domestic livestock in India

Screening of the 306 DNA samples for the MAP bio-type was carried out using IS*1311* PCR_REA, and 97.0% samples were 'Indian Bison Type'. However, only 3.0% DNA samples were 'Cattle Type' (Figs 2 and 3). Goats and sheep were exclusively infected with 'Indian Bison Type' bio-type,

Table 5. Disease-wise percent mortality in farm goatherds and sheep flock at CIRG, Mathura (1988–2013)

Causes of deaths/ breeds	Goat farms							Sheep farm	Total (goats and sheep)
	Breeding units			Experimental units			Subtotal	Breeding unit Muzaffar Nagari	
	Barbari	Jamunapari	Jakhrana[d]	Unit I	Unit 2	Unit 3			
JD	6.7(165)	10.8 (221)	23.5 (25)	17.4(106)	8.4 (52)	20.2(141)	10.9(710)	3.0 (47)	9.4 (757)
Suspected for JD[a]	8.3 (205)	11.1 (227)	29.2 (31)	12.4 (76)	7.4 (46)	4.0 (28)	9.4 (613)	9.9(151)	9.5 (764)
Subtotal A	15.1 (370)	22.0 (448)	52.8 (56)	29.8 (182)	15.8 (98)	24.2 (169)	20.3 (1323)	12.9 (198)	18.9 (1521)
Other infectious diseases[b]	54.6(1334)	40.3 (818)	30.1 (32)	35.4 (216)	37.6 (233)	41.1 (287)	44.9 (2920)	37.9 (578)	43.5 (3498)
Other noninfectious diseases[c]	30.1 (737)	37.5 (762)	16.9 (18)	34.6 (211)	46.5 (288)	34.5 (241)	34.7 (2257)	49.1 (749)	37.4 (3006)
Subtotal B	84.8 (2071)	77.9 (1580)	47.1 (50)	70.1 (427)	84.1 (521)	75.7 (528)	79.6 (5177)	87.0 (1327)	81.0 (6504)
Total (*n*) Necropsies	2441	2028	106	609	619	697	6500	1525	8025

JD, Johne's disease (MAP infection).
Figures in parenthesis are numbers.
[a]Weakness & diarrhoea.
[b]Pneumonia, septicaemia, colibacillosis, coccidiosis, enteritis.
[c]Toxaemia, acidosis, cold shock, predation etc.
[d]Jakhrana breed – 2004–2013, Unit 1 (Nutrition experiments, Barbari breed) 1995–2013, Unit 2 (Physiology experiments, Sirohi breed) 1998–2013, Unit 3 (Health experiments, Barbari breed) 1988–1997 & 2005–2013.

Table 6. Percent bio-load of MAP in farm goatherds and sheep flock at CIRG, Mathura (2011–2013) on the basis of causes of culling

Species / Animal Farms / Causes of culling/Breeds	Goat farms						Sheep farm	Total (goat and sheep)
	Breeding units			Experimental units			Breeding nuits	
	Barbari	Jamunapari	Jakhrana	Unit I	Unit 2	Subtotal	Muzaffarnagri	
Johne's disease	9.5 (4)	0	25.0(11)	7.6(1)	7.1 (3)	10.0(19)	2.2(1)	8.5 (20)
Suspected JD[a]	16.6 (7)	32.6(16)	22.7 (10)	69.2 (9)	28.5 (12)	28.4 (54)	40.9(18)	30.7 (72)
Subtotal	26.1 (11)	32.6 (16)	47.7 (21)	76.9 (10)	35.7 (15)	38.4 (73)	43.1 (19)	39.3 (92)
Other diseases	73.8 (31)	67.3 (33)	52.2 (23)	23.0 (3)	64.2 (27)	61.5 (117)	56.8 (25)	60.6 (142)
Total[b]	(42)	(49)	(44)	(13)	(42)	(190)	(44)	(234)

JD, Johne's disease.
Figures in parenthesis are numbers.
[a]Weak, stunted and un-thrifty.
[b]Total losses due to diseases.
[c]Jakhrana breed (2004–2013), Unit 1 – Nutrition experiments (1995–2013), Unit 2 – Physiology experiments (1998–2013). 8

irrespective of the geographical region of the country (Table 8).

Discussion

Johne's disease is endemic, and bio-load of MAP is increasing world-wide (NAHMS, 1997). In India, JD has impact on the population demography of the four domestic livestock species. Population of buffaloes, goats and sheep, which can be salvaged for meat, has shown an increasing trend in the last 50 years. Due to the ban on cow slaughter, the salvage value of cows is zero; therefore, cow population showed a declining trend in the past few years (FAO, 2013). The cow is considered holy in Indian mythology, and the anti-viral and anti-bacterial properties of cow urine and dung has long been established (Samhita, 1885; Basak et al., 2002; Jarald et al., 2008). India is the leading milk producer in the world (More, 2009), but this achievement is number driven (Birthal et al., 2006), and native breeds of Indian livestock are known for their low per-animal productivity (Bardhan and Sharma, 2013).

The present study focuses on providing evidence, trends and current status of bio-load of MAP in the native livestock species of goats, sheep, cattle and buffaloes from farm

Table 7. Percent bio-load of MAP infection in goats and sheep farms at CIRG, Mathura using microscopy of tissues of goats slaughtered for meat production and necropsied (2011–2013)

Type of goats and causes of deaths	Goats slaughtered for meat production		Goats died and necropsied			
	Apparently healthy goats (82)		Johne's disease (5)		Other diseases (20)	
Tests/Tissues	MLN (82)	INT. (82)	MLN (5)	INT. (5)	MLN (20)	INT. (20)
Microscopy	31.7 (26/82)	32.9 (27/82)	40.0 (2/5)	60.0 (3/5)	25.0 (5/20)	30.0 (6/20)
PCR	10.8 (4/37)	10.8 (4/37)	ND	ND	ND	ND

JD, Johne's disease; ND, not done; MLN, mesenteric lymph nodes; INT, intestines near ileocaecal junction.
Figures in parenthesis are numbers (Positive/Total).

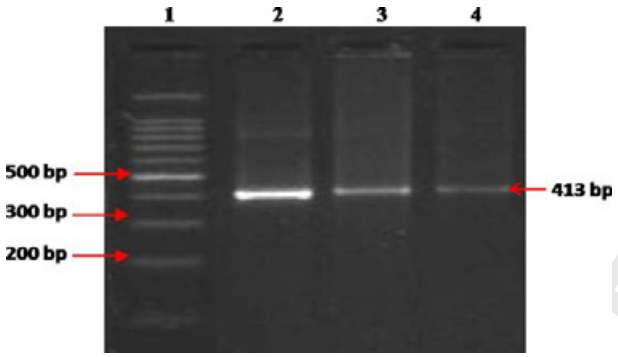

Fig. 2. *Mycobacterium avium* subspecies *paratuberculosis* specific amplicons (413 bp) using IS*900* specific primers. Lane 1: 100 bp ladder, lane 2: Positive control (MAP DNA), lane 3–4: DNA samples. 13

and farmer's animals in different geographical regions of country. A significantly increasing trend in the bio-load was seen between time zone A and B ($P < 0.03$), between B and C ($P < 0.03$) and between A and C ($P < 0.001$).

On the basis of this study, livestock species-wise, the average bio-load of MAP was found to be lowest ($P < 0.05$) in goats, compared with sheep, cattle and buffaloes (Table 1). However, it cannot be ruled out based on these data that the lower bio-load in goats is host-specific phenomena because the sampling was not random; source of the sample (severity of infection in particular herd) received may greatly influence the outcome of the results. Despite the high slaughter rate of buffaloes, goats and sheep, the cumulative average percent bio-load of MAP revealed an increasing trend and was doubled from 11.6% in 1985 to 23.3% in 2013 (Table 1). The study reported, rapid rise in the average bio-load of MAP in four domestic livestock species from 27.0% (2001–2010) to 38.8% (2011–2013). Stress factors like high animal density, dismal sanitary conditions, degradation of nutrition resources (quality and quantity), poor health care and concurrent disease conditions (high parasitic load) may probably be the cause of the increasing trend in the latest bio-load (2011–2013) in all the domestic livestock species. Therefore, a national policy for the control of JD is thus urgently required.

Table 8. Livestock species and state-wise bio-profile of MAP using IS1311 PCR-REA (2004–2013)

Species	State	IS*900* positive DNA (*n*)	MAP bio-type (IS*1311* PCR_RE)	
			Indian Bison type (*n*)	Cattle type (*n*)
Goats	1. Uttar Pradesh	87	87	NIL
	2. Himachal Pradesh	4	4	NIL
	3. Madhya Pradesh	7	7	NIL
	4. Assam	3	3	NIL
	5. Rajasthan	6	6	NIL
Sheep	1. Uttar Pradesh	15	15	NIL
	2. Tamil Nadu	20	20	NIL
Cattle	1. Uttar Pradesh	120	116	4
	2. Punjab	12	10	2
	3. Himachal Pradesh	6	6	NIL
Buffaloes	1. Uttar Pradesh	11	10	1
	2. Punjab	13	11	2
	3. Tamil Nadu	2	2	NIL
Grand total		306	297 (97.0%)	9 (2.9%)

In India, bio-load of MAP in cattle was consistently high (47.0, 31.0 and 50.3% in time zones, 2001–2005, 2006–2010 and 2011–2013, respectively). In the absence of National policy for the control of MAP in the domestic livestock population of the country, the population of low and unproductive cows has increased in the country, which probably may have contributed to the phenomena of increasing trend in the bio-load of MAP in the country. Bovine Johne's disease has been a major health problem in the dairy cattle herds of developed countries, wherein, 70.4, 55.0 and 47.0% prevalence was reported from US (Lombard et al., 2013), Netherlands (Muskens et al., 2000) and Denmark (Nielsen et al., 2000), respectively.

Geographical zone-wise, bio-load of MAP was significantly higher ($P < 0.05$) in Central zone (50.5%) compared with South, West, East and North zones. The highest bio-load in the Central zone may be due to the screening of more number of samples from clinical cases

LOW RESOLUTION COLOR FIG

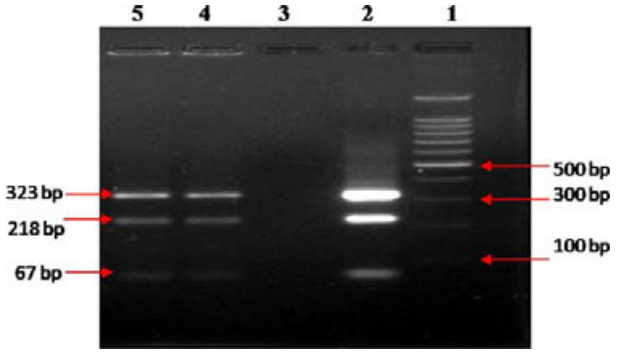

Fig. 3. IS*1311* PCR_REA analysis. Lane 1: 100 bp DNA ladder, lane 2: Positive control, lane 3: Negative control, lane 4 and 5: Digested DNA sample ('Indian Bison Type'). 14

of JD. Bio-load of MAP was moderate in Western zone (30.2%). Of the three states (Rajasthan, Gujarat and Maharashtra) in the Western zone, bio-load was highest in Gujarat (47.5%) followed by Rajasthan (40.3%) and Maharashtra (16.2%). Samples screened from the Western zone were driven from goats and sheep reared under extensive management system. Of the 297 samples of goats screened from Eastern zone, bio-load was higher in Odisha (33.7%) and Assam (33.3%) compared with West Bengal (16.0%). Of the 999 samples (mainly goats and sheep) screened from the South zone, bio-load was highest (87.8%) in Kerala (suspected farm goats) compared with goats and cattle farm in Karnataka (47.2%) and was lower (28.9%) in farms of sheep and cattle from Tamil Nadu. Gupta et al. (2012) reported 15.1% of prevalence of JD in the cattle population of South-western Bangalore, Karnataka, using ELISA. Higher bio-load in Kerala and Karnataka was due to the screening of Malabari farm goats suffering from clinical JD. Of the 17 474 samples screened from North zones, bio-load of MAP was lowest (20.6%) in all the zones of the country. This may be due to screening of large number (17 474) of goats in last 28 years. In time zone A (1985–2000), only goats were screened, and during this period, microscopy was the main screening test. Culture was used on limited samples in this period as it took a long time. ELISA test was under standardization in this period using wild type antigen by harvesting MAP from intestines of a clinical case of JD. Within North zone, bio-load was highest in Himachal Pradesh (67.2%). This was due to screening of clinical cases of JD from HP. Bio-load was moderate (24.6–25.8%) in cattle and buffaloes population of Haryana and Punjab (Table 2). This was mainly due to screening of young apparently healthy animals and better availability of feeding resources as both these states are leading states in agricultural production. Therefore, availability of agriculture by-products was high, which are utilized by ruminants (Pachauri, 2012). This clearly established the role of nutrition in pathogenesis of JD.

Presently, the country lacks reliable and sensitive field-based tests for the diagnosis of JD. Johnin, which had been extensively used in the past, suffers from poor sensitivity and high variability in specificity (Kalis et al., 2003). Lack of field-based diagnostic test and data on National prevalence of JD are primarily responsible for the absence of priority and policies for the control of JD in the country. As per this study, microscopy and ELISA can be adopted as 'screening tests' for the diagnosis of JD and can be easily performed in the 'field laboratory', and hence may be used in designing the National strategy for the control of JD at the national level (Singh et al., 2009, 2013b).

The choice of the diagnostic tests for the diagnosis of MAP is crucial for the success of the control programs. The choice of the diagnostic test however, usually depends on the purpose and should invariably involve multiple tests in case of chronic infections like MAP. In the present study (1985–2000), microscopy and culture were mainly used for the screening of animals at CIRG, Mathura. Comparative evaluation of the four tests in the present study revealed the highest percent positivity in ELISA (35.1%), followed by PCR (26.6%), microscopy (20.0%) and culture (10.6%) (Table 3).

ELISA was standardized for the first time in 1985–1990, using wild type antigen, harvested directly from the intestines of an advanced clinical case of JD in the Jamunapari goat (Singh et al., 1999). Later (1996–2000), sonicated semi-purified soluble protoplasmic antigen prepared from native strain 'S 5′ of MAP, which was later characterized as 'Indian Bison Type' bio-type (Sohal et al., 2009), was used in the ELISA test. In 2004–2005, an indigenous ELISA test was standardized and converted into 'ELISA kit' based on 'likelihood ratio' method of Collin (2002). Wherein, OD values were transformed as ratio (sample to positive ratio) based on their equation and the values are distributed on 0–10 scale (0.00–0.09, 0.10–0.24, 0.25–0.39, 0.40–0.99 and 1.00–10.0 and corresponding status of JD is negative, suspected, low positive, positive and strong positive, respectively). Of these, animals in the strong positive category are taken as positive. Therefore, 'indigenous ELISA kit' along with faecal microscopy can be very good test combinations for the mass screening and for diagnosis of MAP infection (Singh et al., 2008a). Indigenous ELISA kit developed by CIRG, Mathura (Singh et al., 2007) has shown good correlation with fecal culture (Singh et al., 2007). This kit, on comparison with commercial kits, exhibited similar sensitivity and specificity (Singh et al., 2009). Use of 'indigenous ELISA kit' in the present studies improved the detection rate of MAP in the test samples. There was considerable improvement in the detection rate of bio-load of MAP from 28.9% (2001–2010) to 47.0% (2011–2013) (Table 3).

Similar to the above findings, Kurade et al. (2004) reported lymphocyte stimulation test for cellular immune response and ELISA for humoral immune response overall sensitivities of 65.0 and 42.0%, respectively, in sheep with different types of pathology, but when employed together could detect about 88.0% of infected animals. Similarly, Sonawane and Tripathi (2013) reported higher sensitivity of ELISA (77.7%), compared with culture (50.0%), IS900 PCR (55.2%) in tissues with both pauci and multibacillary conditions in sheep. Many workers have used ELISA as screening test for the estimation of bio-presence of MAP in domestic livestock (Kumar et al., 2006; Mercier et al., 2010; Gupta et al., 2012).

Although culture is 'Gold standard' for the diagnosis of MAP infection, it was not useful in the routine screening of animals, as it takes a very long time for the appearance of visible colonies and short shelf life of mycobactin J (personal observation). Therefore, culture has value only as 'confirmatory test', and that too, on limited samples.

Microscopy was also useful being simple, repeatable and easy to standardize in a 'field laboratory', and therefore, has potential to replace 'allergic tests' as 'field laboratory test' (Singh et al., 2013b). Use of microscopy over the years showed increased bio-load of MAP from 9.1, 17.3, 23.8, 67.5 and 32.4% at five -yearly intervals of 1985–1990, 1996–2000, 2001–2005, 2006–2010 and 2011–2013, respectively.

IS*900* PCR was introduced in 2001 as quick confirmatory test, but was costly. IS*900* PCR detected the highest number of animals as positive (35.8%) in 2001–2010, in comparison with microscopy (32.6%), ELISA (28.9%) and culture (9.6%). This is due to the high sensitivity of IS*900* PCR as diagnostic test. Wells et al. (2006) also reported that PCR assay has higher sensitivity for detection of heavy faecal shedder than ELISA in dairy cattle. Similarly, Sivakumar et al. (2005) and Vinodhkumar et al. (2013), reported high sensitivity of IS*900* PCR compared with tissue culture in buffaloes and sheep, respectively. As IS*900* PCR is a confirmatory test, therefore high positivity in 2001–2010, showed high endemicity of MAP infection in the domestic livestock population of the country.

Microscopy and indigenous ELISA could be good screening tests individually or in combination, and use of IS*900* PCR increased specificity and sensitivity of detection of MAP. Percent positivity of microscopy and ELISA was higher compared with IS*900* blood PCR in goats and sheep (both in farm and farmer's animals). Whereas, all the three tests exhibited high percent positivity in farmer's cattle herds (Table 4). In the absence of user friendly 'indigenous diagnostic kits', each of the four tests used in this study showed limitations as individual tests and variations in results were due to variability in respect to animal species and population, type of samples, time of sampling, diagnostic test used, person to person etc., in the last 28 years. Better performance of the tests depended on the standardization of the tests, harmonization of reagents, continuous training of the laboratory staff, individual skills of the user etc.; therefore, it is essential to accredit the laboratories for the screening of samples for MAP infection in the country and to set up a national referral laboratory. The apex body (OIE) looking after global animal health is yet to take note of high bio-load of MAP in the domestic livestock population and resulting losses in developing and poor countries.

Endemicity of JD disease added to the stress, which often combined with high load of parasites (coccidiosis, hemonchosis, tapeworms, lice etc.) leading to higher susceptibility and early development of clinical symptoms. Endemicity of MAP in herds and flocks inflicts many types of losses in animal-based production system and which may include both direct and indirect losses. These losses usually go unnoticed. Higher bio-load of MAP was correlated with other losses in productivity (short productive life by early culling, and low per-animal productivity of meat and milk), higher morbidity and higher culling rate in goats and sheep farms located at CIRG, Makhdoom.

In 25 years (1988–2013), a total of 8025 goats and sheep died due to various reasons at the farms located at CIRG, Makhdoom. Of the total (8025) goats and sheep that died, deaths (direct losses) due to JD were 10.9% in goats compared with Muzaffarnagri sheep, where these losses were substantially low (3.0%). In goats, deaths due to JD varied from 6.7 to 23.5% in different breeds and units of goats. On an average in the last 25 years, 9.4% goats and sheep were lost due to JD in the farm herds and flock. Deaths due to suspected JD cases were 9.4 and 9.9% in goats and sheep, respectively in the last 28 years (Table 5). Within goat breeds located at CIRG, Mathura, the study showed that dairy goats (Jakhrana and Jamunapari) were more susceptible to MAP infection compared with meat breeds (Sirohi and Barbari). This may be due to increased stress due to high milking ability and from shifting of these goats from farmer's herd to farm herds in 2005 at CIRG, Mathura, where disease was endemic in Barbari and Jamunapari breeds of goats since establishment of goat farms in 1976. Losses, due to the suspected JD (animals exhibited clinical symptoms of unthriftiness, poor in body condition, weak, diarrhoeic and stunting) but were negative for acid-fast bacilli (AFB) in microscopy, were also high in these farms. Goat herds at CIRG also suffered with other major problems of weakness and stunting (all age groups) and coccidiosis, in young age.

Besides goat culled due to JD, the suspected animals were also regularly screened for the presence of MAP (shedding) bio-load in faecal microscopy and were found negative. Goats and sheep shedding MAP in faeces (positive in microscopy) and those suspected for JD and

negative in microscopy were also culled from the stock. Average losses due to culling of goats and sheep shedding MAP bacilli in faeces in time zone C (2011–2013) were high (10.0%) in goats compared with Muzaffarnagri sheep (2.2%). In different goat breeds, the culling rate due to JD varied from 9.5 to 25.0% in farm animals at CIRG, Mathura in time zone C (2011–2013) (Table 6). Here again losses due to culling were high in Jakhrana breed of goats. Jakhrana goats suffered from higher milking stress. Culling is an important tool in the management of livestock farms, wherein animals infected with incurable MAP infection were removed to manage JD infection in farm herds and flocks. JD being endemic, the immune system of animals was weak and damaged as MAP grows in lymph nodes and animals lose protein due to diarrhoea and low absorbance of digested food in view of the damage of intestinal mucosa and are in state of negative energy balance; therefore, animals become susceptible to other infections. Animals from young age are more prone to viral infections causing pneumonia, enteritis, *Escherichia coli* and coccidiosis infections. In such animals JD starts developing early and soon become clinical cases of JD. Many times, these young animals in general and adult ones in particular, although negative for MAP infection but were weak, unthrifty, stunted, losing body weights were negative for MAP infection in faecal microscopy, but were also culled as it was uneconomical to retain them in the herds/flock. Losses due to culling of these suspected clinical cases (suspected suffering from chronic infections like JD) were high in sheep (40.9%) compared with goats (28.4%) in CIRG farms.

Bio-typing of MAP strains was started with help of Australia and Spain in 2000 and 2004 (Sevilla et al., 2005) and was later adopted at CIRG, Mathura in 2004 and has since been continued to study the molecular epidemiology of MAP infection in different species of domestic livestock located in different geographical regions of the country. Molecular epidemiology of MAP infection carried out from 2004–2013 showed that 'Indian Bison Type' was the major bio-type (97.0%) infecting goat, sheep, cattle and buffalo population of the country. Goats and sheep were exclusively infected with 'Indian Bison Type' irrespective of the geographical region. 'Cattle Type' was recovered from 3.0% cases in cattle and buffaloes. Other studies also revealed the presence of 'Indian Bison Type' bio-type of MAP in the biotic (rabbits, blue bulls, deer, bison, primates and human beings) and abiotic (soil, water, raw milk, pasteurized milk and milk products) environment of the country (Singh et al., 2008b, 2010a, 2011, 2012a; Shankar et al., 2010). These studies showed the high pathogenicity native strain ('Indian Bison Type') of MAP compared with 'Cattle Type'. The 'Sheep Type' bio-type of MAP was not recovered in our studies. Therefore, these studies confirmed the results of the diagnostics and vaccines developed from native MAP strain ('Indian Bison Type') worked better compared with imported kits and vaccines (Singh et al., 2007, 2009), which are based on 'Cattle Type' bio-type. JD is endemic in the domestic ruminant population world-wide, and therefore needs unified attempts to tackle this major infectious disease plaguing domestic livestock globally and also threatening human life (Naser et al., 2000). As this study is not based on random sampling of herds and flocks, there is a need for well-designed studies to know the national prevalence of MAP infection in the domestic livestock population.

Conclusion

The 28-year (1985–2013) study showed that bio-load of MAP infection was moderately high and Johne's disease was a major disease and endemic in the domestic livestock population of the country. Over the time period of 28 years, the bio-load of MAP in domestic livestock of country had shown an increasing trend. Microscopy and indigenous ELISA tests were found as good screening tests and IS*900* PCR was a quick confirmatory test in the study. Employment of multiple tests proved to be useful for the screening of animals against MAP infection. Species-wise, goats and cattle had higher susceptibility compared with buffaloes and sheep. With respect to management system, there was no difference in the bio-load of MAP between farm (semi-intensive management) and farmer's (extensive management) herds and flocks. Besides direct losses (deaths and culling) caused by JD, indirect losses as result of mortalities and culling due to suspected cases of JD were also high in the farm herds and flocks of CIRG, Mathura, which were endemic for JD. Bio-load of MAP was high in farm animals even in apparently healthy young animals that were slaughtered for meat production and in animals that died due to other diseases. Due to inherent difficulties in diagnosis of MAP infection in animals, the present study showed usefulness of multiple tests for the screening of animals. 'Indian Bison Type' was the major bio-type infecting domestic livestock cutting across geographical and species barrier. In view of the increasing trend in the bio-load of MAP in domestic livestock of country, there is urgent need to estimate the 'National prevalence of MAP infection' and also initiate a national Johne's disease control program on priority. Control of JD will not only help to improve per-animal productivity of the domestic ruminants but also reduce threat to human population.

Conflict of Interest

No conflict of interests to declare.

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USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

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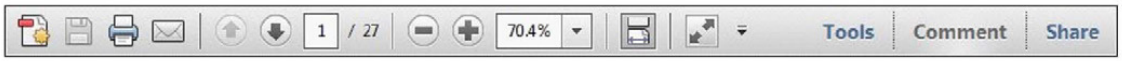
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



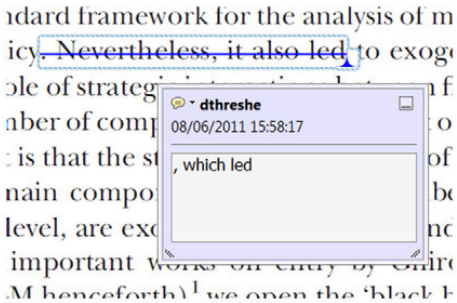
This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

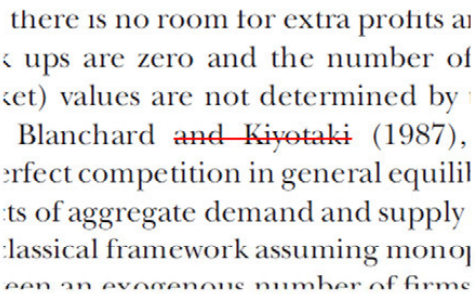


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

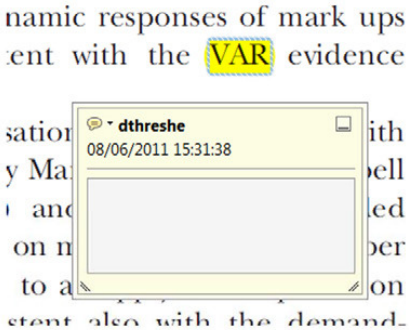


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

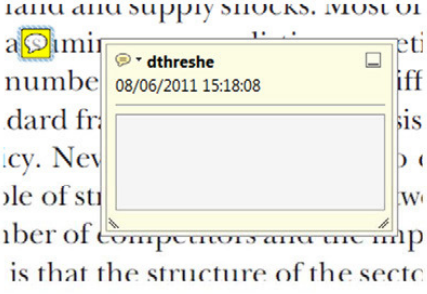


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

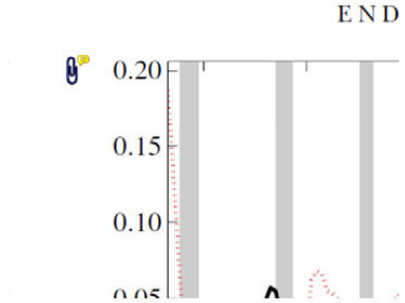


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

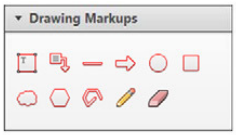
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.