Present study was aimed to estimate the bio-incidence of Bovine Johne’s disease in native population of buffaloes (Murrah, Zaffarabadi and Bhadawari) in dairy farms and farmer’s herds located in Central and North India. Total 156 serum samples were screened from a dairy herd in Kiratpur (Malwa region), and 41.0% buffaloes were positive for Mycobacterium avium subsp. paratuberculosis (MAP) infection using “Indigenous ELISA kit”. In another study, 101 young males (farmer’s herds) from the native tract (Jind / Rohtak, Haryana) of Murrah breed were screened and 80.1% were reported positive by kit. In a farm herd of Bhadawari buffaloes in its native tract (Etawah district in Uttar Pradesh) and dairy herd of Murrah buffaloes procured from native tract and kept in Mathura district of Uttar Pradesh, in the two farms 20.0% buffaloes were screened and 71.4% were positive by fecal Microscopy. Study reported high bio-incidence of BJD in native breeds of buffaloes in Malwa region of Central India, Etawah and Mathura district of Uttar Pradesh, and Jind / Rohtak (Haryana) regions of North India, despite high slaughter rate, for meat harvesting. Goat based 'Indigenous ELISA kit' was employed for screening since commercial kits were costly for the screening of buffaloes. Moreover Imported 'ELISA kits' were based on PPA from 'Cattle Type' biotype, a different strain from other geographical regions / country. ‘Indigenous ELISA kit’ using native ‘Indian Bison type’ biotype was efficient, specific and cost effective ‘mass screening’ test to estimate bio-incidence of BJD in large population of buffaloes at National level.

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ABSTRACT

Present study was aimed to estimate the bio-incidence of Bovine Johne’s disease in native population of buffaloes (Murrah, Zaffarabadi and Bhadawari) in dairy farms and farmer’s herds located in Central and North India. Total 156 serum samples were screened from a dairy herd in Kiratpur (Malwa region), and 41.0% buffaloes were positive for Mycobacterium avium subsp. paratuberculosis (MAP) infection using ‘Indigenous ELISA kit’. In another study, 101 young males (farmer’s herds) from the native tract (Jind / Rohtak, Haryana) of Murrah breed were screened and 80.1% were reported positive by kit. In a farm herd of Bhadawari buffaloes in its native tract (Etawah district in Uttar Pradesh) and dairy herd of Murrah buffaloes procured from native tract and kept in Mathura district of Uttar Pradesh. In the two farms 20.0% buffaloes were screened and 71.4% were positive by fecal Microscopy. Study reported high bio-incidence of BJD in native breeds of buffaloes in Malwa region of Central India, Etawah and Mathura (Uttar Pradesh) and Jind / Rohtak (Haryana) regions of North India, despite high slaughter rate, for meat harvesting. Goat based 'Indigenous ELISA kit' was employed for screening since commercial kits were costly for the screening of buffaloes. Moreover Imported 'ELISA kits' were based on PPA from 'Cattle Type' biotype', a different strain from other geographical regions / country. ‘Indigenous ELISA kit’ using native ‘Indian Bison type’ biotype was efficient, specific and cost effective ‘mass screening’ test to estimate bio-incidence of BJD in large population of buffaloes at National level.

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1 Introduction

Buffaloes are present in 44 countries over four continents (Asia, Africa, Latin America, and Europe). Buffalo population increased at annual growth rate of 3.3%, (135 million in 1991 to 165 million in 2007); however, major population is concentrated in four countries, India (61%), China (20%), Pakistan (13%) and Egypt (2.2%) (Soliman, 2009). Buffalo rearing plays significant role in alleviation of poverty, provides gainful employment and livelihood security to millions of individual farmers and commercial dairy farmers in rural communities of India (Sivakumar et al., 2006).

Trade of buffalo meat and milk is vital in most developing Asian and African countries. Though buffaloes are primarily reared for milk production but have also emerged as important meat animal in India, since it has good salvage value. India at 127.8 million tonnes in 2011-12 (DADF, 2013) is the leading milk producer country in the world and buffalo’s contribution at 62.35 million tonnes was 51.1% of the total milk produced in the country. Buffaloes are also important source of meat and produced 0.80 million tonnes of meat, which was 16.5% of the total meat produced (BAHS, 2012). Buffalo population of the country has seen consistent increase from 43.4 million in 1951 to 109.0 million in 2013 (FAO, 2013). Slaughter rate of low productive buffaloes is very high to meet ever growing demand of meat for export and internal consumption. Morbidity is also very high in buffaloes and is dispersed over long period of times, thus production losses usually go unnoticed and never estimated in India despite low per animal productivity (Barbaruah & Joshep, 2008). This is mainly due to presence of chronic infections, like Bovine Johne’s Disease (BJD). ‘Indigenous ELISA based diagnostic kit’ has proven to be efficient and most widely validated kit in the country for the detection of this disease (Singh et al. 2016). Hence the kit was used in this study to estimate the status of Johne’s disease in buffaloes, which could not be estimated earlier in the native population of riverine buffaloes.

Present study estimated status of BJD in native population of the buffaloes (Murrah, Jaffrabadi, Bhadawari) in a dairy farm (Itarsi, MP), in the Malwa region of Central India, farmer’s herds (Murrah; Jind / Rohtak, Haryana), using this highly sensitive goat based ‘Indigenous ELISA kit’. Two buffalo farm herds (Bhadawari breed native of Etawah and Murrah breed kept in the Mathura district) in Uttar Pradesh were screened in parallel to compare bio-incidence by estimating shedding of MAP in the faces of buffalo herds.

2 Materials and Methods

2.1 Animals and samples

Three major native breeds of buffaloes from 4 different places of North and Central India farm and farmer’s herds were screened to estimate bio-incidence of MAP in highly valuable and fast growing species (buffaloes) as compared to other three species (goats, sheep and cattle) of domestic livestock. Samples (serum and feces) collected were stored at -20°C before processing.

2.1.1 Buffalo farm herd of State livestock board (Madhya Pradesh)

A large buffaloes farm of native breeds (Murrah, Jaffrabadi and Bhadawari) is maintained at Kiratpur in Itarsi district of Madhya Pradesh. Buffaloes were provided optimum to intensive feeding (green fodder, dry straw called ‘bhussa’ along with high quality of mineral mixture). Despite good feeding and better management conditions, a good number of buffaloes were identified as suspected for Bovine Johne’s disease (BJD) on the basis of lower body weight and, visibility of ribs from distance, reduced productivity at regular intervals. These identified buffaloes along with apparently healthy were sampled for the screening of BJD. A total of 156 serum samples were collected randomly from suspected and apparently healthy buffaloes of all the three breeds (Murrah, Jaffrabadi and Bhadawari), between March and September 2015.

2.1.2 Buffalo farm herd of State livestock board (Uttar Pradesh)

A farm herd of Bhadawari breed of buffaloes located in the native tract Etawah city of Uttar Pradesh as part of scheme of State Animal Husbandry Department to save the precious germplasm of this breed. Farm consisted of 75 buffaloes (all age groups), where management conditions were sub-optimal in terms of feeding, management and hygiene. Condition of 50.0% animals was normal; others looked unthrifty, poor in body condition, suffering with chronic weight loss, weakness and symptoms of diarrhea. Of these 75 animals, fecal samples were collected from 10 buffaloes randomly and examined microscopically by acid fast staining.

2.1.3 Murrah breed of buffaloes (farmer’s herds), Etawah, Uttar Pradesh

As part of scheme of Animal Husbandry Department of Uttar Pradesh to upgrade local animals and boost milk production of the state, young males of pure Murrah breed of buffaloes are distributed time to time by purchasing animals from native tract in Jind and Rohtak districts of Haryana. Blood samples of 101 such young male buffalo calves were collected from animals belonging to different farmers and were screened for MAP infection using indigenous ELISA kit.

2.1.4 Farm herd of Murrah, buffaloes, Mathura, Uttar Pradesh

A new dairy farm consisting of Murrah breed of buffaloes along with Holstein Frisian cattle was established in Mathura district after purchasing buffaloes from native tract in Haryana in 2012-13. Dairy herd consisted of 47 buffaloes of all age groups, where management conditions were good in terms of
feeding, management and hygiene. Except few animals, condition of buffaloes was normal / good. Few buffaloes were weak in body condition, suffering with chronic weight loss and diarrhea. Of these 47 animals, fecal samples were collected from 7 buffaloes randomly and examined microscopically by acid fast staining.

2.2 Screening tests

Buffalo has been a integral part of livestock agriculture in Asia for over 5000 years (Nanda, 2003). Efficient Indigenous ELISA kit [semi-purified PPA from native biotype (Indian Bison Type) of goat origin] were with respect to commercial ELISA kits based on PPA from MAP strains (Cattle Type) from different geographical regions / countries.

2.2.1 Indigenous ELISA kit (i_ELISA) using goat based antigen

Soluble ‘semi-purified Protoplasmic antigen’ (sPPA) harvested from native strain (S 5) of MAP characterized as ‘Indian Bison Type’ biotype (isolated from a terminal case of Johne’s disease in a Jamunapari goat) (Sevilla et al., 2005; Singh et al., 2007a; Sohal et al., 2009) was used in the ‘Indigenous ELISA kit’ for the screening of buffaloes (Singh et al., 2007b). sPPA was standardized at 0.1 microgram per well of the microtiter plate. The dilution of Serum samples was used as 1:50 dilution. A 1: 2500 dilution of anti-bovine horseradish peroxidase conjugate (Sigma Aldrich, USA) was used to detect antigen-antibody complex. Known serum samples from culture positive and negative of cattle were used as positive and negative controls, respectively.

2.2.2 Interpretation

Optical density was measured at 450 nm and OD values were transformed and expressed as sample-to-positive (S/P) ratios. Serum samples in strong positive and positive range of S/P ratio were categorized (type 1) as positives in i_ELISA (Table 1).

S/P percent = [(Sample OD – NegativeOD)/(Positive OD - NegativeOD)] X 100.

Table 1 Sample to positive ratios and status of Johne’s disease on the basis of likelihood ratio (Collins, 2002).

| S.No. | S/P Ratios       | Status of Johne’s disease |
|-------|------------------|---------------------------|
| 1     | 0.00 to 0.09     | Negative                  |
| 2     | 0.10 to 0.24     | Suspected or Borderline   |
| 3     | 0.25 to 0.39     | Low Positive              |
| 4     | 0.4 to 0.99      | Positive                  |
| 5     | 1.0 to 10.0      | Strong Positive           |

2.3 Ziehl Neelsen staining

Microscopy (Ziehl Neelsen staining) was performed to detect acid fast M. avium subspecies paratuberculosis in fecal sample (Sahzad et al. 2015; Chaubey et al. 2016). Briefly, two gram of fecal sample was homogenized in 1X PBS and concentrated by centrifugation at 4500 rpm for 45 min at room temperature. Supernatant was discarded and smear were heat fixed, stained by Ziehl Neelsen method and examined under the microscope for pink colour (acid fast) short rods indistinguishable to MAP was considered positive.

3 Results

When 'type I’ reactors (buffaloes in positive and strong positive categories considered as positive) were considered as positive for MAP infection, of 156 buffaloes screened, 41.0% (64) and 58.9% (92) were positive and negative in i_ELISA, respectively. When low positive buffaloes were also considered as positive (type II reactors), 85.8% (134) and 14.1% (22) buffaloes were positive and negative in i_ELISA, respectively (Table 2).

On the basis of S/P ratio, in i_ELISA, 0.6, 40.3, 44.8, 11.5 and 2.5% buffaloes were in the strong positive, positive, low positive, suspected and negative categories with respect to the bio-incidence of MAP infection. Of the 101 serum samples of young Murrah buffaloes screened, 20 (19.8%) were negative and rest 81 (80.1%) were positive for MAP infection [1 (0.9%) in strong positive range and 80 (79.2%) in positive range] using i_ELISA kit. Of the 10 fecal samples (Bhadawari buffaloes, Etawah) screened by microscopy, 2 (20.0%) were positive (shedding) for acid fast bacilli indistinguishable to MAP.

Table 2 Bio-incidence of Johne’s disease in buffaloes of Malwa region (Central India) using goat based indigenous - ELISA (n=156 samples).

| ‘Reactor type’ | Status in Indigenous- ELISA test |
|---------------|----------------------|
|               | Negative (N) | Suspected (S) | Low Positive (LP) | Positive (P) | Strong Positive (SP) |
| Type I        | 4 (2.5)       | 18 (11.5)     | 70 (44.8)         | 63 (40.3)    | 1 (0.6)             |
| Type II       | 92 (58.9)     |               |                   | 64 (41.0)    |                    |
|               | 22(14.1)      |               |                   | 134 (85.8)   |                    |

Table 2 Bio-incidence of Johne’s disease in buffaloes of Malwa region (Central India) using goat based indigenous - ELISA (n=156 samples).
Figure 1 Corrugation and thickening of Intestine due to MAP infection in buffalo.

Figure 2 (A&B) Detection of MAP bacilli in intestine of buffaloes by microscopy (ZN staining).

Figure 3 Detection of MAP bacilli in mesenteric lymph nodes of buffaloes by microscopy (ZN staining).
Of the 7 buffaloes (Mathura) sampled, 5 (71.4%) were positive (shedding MAP bacilli) in microscopy. Autopsy examination of one buffalo died during the study period showed advanced lesions (corrugation and thickening of intestines) of JD (Figure 1) and microscopic examination of intestine and MLN tissues showed acid fast bacilli indistinguishable to MAP (Figure 2A, 2B and 3).

4 Discussions

Though India is highest milk producer in the world, per capita milk availability has reached to 290 g / day (2011-12) was higher than world average of 284 gms / day. Value of 14, 75, 526.01 MT of buffalo meat exported in 2014-15 was Rs. 29,282.60 crores (APEDA, 2015). It also provides 10.0% of the total draught power of the work animals and also produces 0.52 million MT of skin and hides (DADF, 2013). There are many routes by which MAP reaches to animals and infects others animals. For horizontal transmission, fecal-oral is the main route by which MAP spreads from one animal to other animal and to other species (Sweeney,1992; Greig et al., 1999; Ayele et al., 2002). Transmission of MAP was also reported from semen, in-utero and fetus (Buergerl et al., 2006).

In India, though Johne’s disease was first reported in 1913 but so far it has not been eradicated and continues to be a major health problem in domestic ruminants (Singh et al., 2014a). Nation-wide estimates on bio-incidence of MAP infection in buffalo population are not available. This is mainly due to lack of priority to Johne’s disease and non-availability of the indigenous kits for the diagnosis of MAP infection. Singh et al. (2007b) evaluated i_ELISA kit with commercial purified PPA of Allied Monitor Inc., USA and commercial ELISA kit (Pourquier, France) in goats and sheep with culture and reported that as compared to type II reactors, type 1 reactors in i_ELISA had maximum correlation with culture. Yadav et al., (2008) first time standardized goat based ‘indigenous ELISA kit’ (Singh et al., 2007b), for sero-screening of unproductive buffaloes slaughtered for meat production from Agra region of South Uttar Pradesh (UP) and reported similar high (46.7%) sero-incidence of MAP infection. Using culture of tissues (MLN and large intestine) 48.0% buffaloes were positive and 40% were positive by IS900 PCR. In another large scale sero-survey in buffaloes from North India using this i_ELISA kit, Singh et al., (2008a) reported 28.6% sero-incidence. State-wise sero-incidence of BJD was 40.3, 25.5 and 23.3% in Murrah breed of buffaloes in South UP, West UP and Punjab, respectively. Sero-incidence of MAP was high (86.9%) in young bulls of Hariana breed (Singh et al., 2007c). Sero-incidence in dairy farm bulls from Ludhiana, Punjab was low (14.2%) using this kit, but large number of bulls were on borderline (73.4% type II reactors) (Singh et al., 2007c).

However, sero-incidence in young bulls of Murrah breed in their native tract (Rohtak, Haryana) was 56.0% (Singh et al., 2014b). In a mega study by Singh et al., (2014a) based on multiple tests (microscopy, indigenous ELISA kit, culture and IS900 PCR), 28.3% average bio-load of MAP infection was reported in buffaloes from North India in last 28 years (1985-2013). Sivakumar et al. (2005) reported similar high (70.0 and 30.0%) incidence in buffaloes from North India using PCR and culture. In another large study by Singh et al., (2008a), sero-prevalence of Johne’s disease was 28.6% in buffalo in Northern India. In Uttar Pradesh, of 601 randomly sampled buffaloes, sero-prevalence of MAP infection by Indigenous ELISA kit was 40.3% (16.6% in young and 40.9% adults) and 25.5% (10.5% in young and 26.3% adults) in South and West UP, respectively. In Punjab (Ludhiana), of 699 serum samples screened, sero-prevalence was 23.3% (12.1% in young and 24.4% in adults) in Murrah buffaloes. Indigenous ELISA kit used was proven as a rapid, economic and sensitive test for large-scale screening of buffaloes population against incurable BJD. Sivakumar et al., (2005) estimated sero-prevalence of MAP in buffaloes to be 21.3% in Chennai (South India). In another study, Ziehl-Neelsen’s stained tissue sections revealed acid-fast bacilli in grade-3 and grade-2 buffaloes and acid-fast granular debris were present in grade-1 buffalo. Out of 20 buffaloes, 14 (70%) were positive by IS900 PCR and 6 (30%) were positive by MAP culture (Sivakumar et al., 2006). Singh et al. (2014b) screened 25 young Murrah bulls, 14 (56.0%) were positive for BJD. Sero-incidence of BJD was high in young bulls of Murrah breed in their native tract (Jind, Haryana). Chauhan et al. (2011), reported a case of advance clinical JD (on the basis of history, clinical symptoms and microscopy) in a buffalo from Gujarat suffering from non-treatable diarrhea, weakness and emaciation. Not only in other breeds of buffaloes but also in native breeds (Singh et al., 2013) and cross-bred (Singh et al., 2014c) cattle population of the country, bovine JD is endemic in North India. Mota et al., (2010) using microscopy reported low incidence (5.0%) of BJD in100 buffaloes in a herd in Fernambuco-Brazil having characteristic clinical signs.

Lillini (2002) found 13.3% prevalence of MAP in the Latium region of Italy using PCR in fecal samples of water buffalo herds. Molecular epidemiology studies by Kaur et al. (2011), showed that as compared to ‘Cattle type’ biotype ‘Indian Bison type’ was the predominant (82.0%) biotype in domestic livestock including buffaloes. Georges et al. (2011) estimated 13.1% of water buffaloes were serologically positive for MAP in ELISA and 13.2% were positive in IFN-γ test. They reported significant association between age and sero-positive test results, (P = 0.007, chi square 1 df, 95% confidence). 1400 buffaloes belonging to 71 herds in the Latium region of Italy were screened by Sezzi (2010) using two different commercial ELISA kits (Pourquier, ID. vet). None of the buffalo was found positive by Pourquier kit, whereas in ID.vet kit 3 buffaloes were positive (0.2% prevalence).A study carried out by Desio et al. (2013) on 1350 buffaloes belonging to 56 herds in the Caserta province, of Campania region, Italy. The prevalence of infected buffalo dairy herds was estimated by a commercial ELISA kit of individual blood samples of animals over 24 months of age. On the basis of sensitivity 43%, specificity 99.3% of ELISA test on serum, the resulting true prevalence at animal level and at herd level was 4% (95% CI 3% to 5%) and 74.1% (95% CI 71.8% to 76%). A 4.5% prevalence of MAP...
infection on the basis of suspected lesions in buffaloes with non-significant difference between age groups was reported by Waqas et al. (2015). However, prevalence was found relatively higher in buffaloes of more than 10 years (6.1%) than buffaloes of less than 5 years (3.63%) of age and between 5 to 10 years (3.75%) years of age. Gamberale et al. (2014) reported bio-load of MAP in buffaloes over 12 months were subjected to yearly serological examination by ELISA and positive animals were culled. Yearly raw prevalence obtained was very low (1.0, 2.0 to 0%) during 2009 to 2012. A 5.8% sero-prevalence of paratuberculosis in buffaloes from unorganized dairy farms in Gujarat was reported by Mohan et al. (2009). In rural dairy population, the incidence of MAP was lower than organized dairy farms. Tripathi et al. (2007) screened sera of 320 buffaloes [Central West India (80), Northern India (240)] for MAP infection by Pourquier ELISA kit, did not show antibody prevalence. Similarly Abdellrazeq et al. (2014) in Egypt using imported indirect ELISA kit (SERELISA M. paraTB Ab Mono Indirect ELISA Kit, Synbiotics Corp., Lyon, France) recorded very low sero-prevalence (18.5% of culture positive buffaloes) as compared to fecal culture (68.0%). Similar findings were reported by Yadav et al., in 2008, using cPPA (Commercial PPA) from MAP of bovine origin from Allied Monitor Inc., USA, none of the buffalo was positive, whereas using sPPA from MAP ‘Indian Bison Type’ strain ‘S 5’ of goat origin 46.7% buffaloes were found positive. Similarly in the present study also, of 156 serum samples screened, 41.0% were found positive by ‘Indigenous ELISA kit’.

Therefore the low incidence reported in the above studies from Italy and by Tripathi et al. (2007) in India, were due to use of commercial ELISA kits, wherein the cPPA used was based on MAP isolates from bovine population located in different countries and MAP biotype ‘Bovine type’ may be different from local dominant bio-type like ‘Indian Bison Type’ in India. Therefore for screening of native population of animals one should always use PPA from locally predominant bio-type of MAP infecting the target livestock species. Gupta et al. (2012) also using ELISA kit (from Institut Pourquier, France) reported low sero-prevalence of BJD in cattle showing diarrhea and/or anaemia, at 5 local dairy farms in South-West Bangalore, India. Out of 350 bovine serum samples, 53 (15.14%) were positive and out of 300 pooled milk samples, 55 samples were positive.

Present study revealed that the bio-incidence of MAP in buffalo (Murrah, Jaffrabadi, Bhadawari) population from Malwa region in Central India, Bhadawari buffaloes in Etawah, UP and Murrah in native tract of Jind / Rohtak in Haryana and a dairy farm in Mathura, North India was high. Review of other studies mainly from North India and one study from Chennai (South India) also showed high, sero-incidence of MAP, which calls for urgent measures to reduce MAP infection in buffalo population of the country. Goat based Indigenous ELISA kit was good as herd/farms screening test and has been recommended for screening of Bovine Johne’s disease in domestic buffalo population. Study recommended use of PPA from locally available strains of MAP, in ELISA, rather than using commercial kits based on PPA from MAP strains from different geographical regions.

This is the first report of BJD in farm buffaloes consisting of Murrah, Jaffrabadi and Bhadawari breeds in a large dairy farm in Malwa region of Central India using goat based ‘Indigenous ELISA kit’. Study recommended use of highly sensitive and cost effective ‘indigenous kit’ for the large scale screening of buffaloes at National level in India. This is very important observation of this study and may also benefit other countries.

Ethical Approval

This study has been conducted while complying with all ethical guidelines prescribed by the Indian government and has received ethical approval from the research institute.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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