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Recovery of Indian Bison Type Genotype of *Mycobacterium avium* subsp. *paratuberculosis* from Wild Bison (*Bos gourus*) in India

[1]S.V. Singh, [1]A.V. Singh, [1]P.K. Singh, [1]B. Singh, [2]A.S. Ranjendran and [2]N. Swain
[1]Veterinary Microbiology Laboratory, Animal Health Division,
Central Institute for Research on Goats, Makhdoom, PO-FARAH, District-Mathura (UP), India
[2]Southern Regional Center of Central Sheep and Wool Research Institute,
Mananvanur (Tamil Nadu), India

Abstract: Study aimed to recover and genotype *Mycobacterium avium* subspecies *paratuberculosis* isolates from free-ranging wild bison (*Bos gourus*) found in the Nilgiri hills of Kodai Kanal region of Tamil Nadu (South India). Of the 13 fecal samples screened for the presence of MAP infection, 6 (46.1%) and 8 (61.8%) were positive in microscopic examination and culture, respectively. Further characterization and genotyping of cultures, using IS900 PCR and IS1311 PCR-REA, study first time revealed presence of Indian Bison Type genotype in the bison population of India. More studies may provide link on the evolution of Indian Bison Type genotype which has been widely reported from domestic ruminants in India. Control of MAP infection in bison will be crucial to check transmission to domestic ruminants and conserve fast dwindling population of wild in India.

Key words: *Bos gourus*, bison, *Mycobacterium avium* subsp *paratuberculosis*, IS1311 PCR-REA, Indian bison type genotype, ruminants

INTRODUCTION

Johne's Disease (JD) or paratuberculosis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in animals is a chronic wasting disease characterized by granulomatous enteritis leading to progressive weight loss and death (Whitlock and Buergelt, 1996). MAP has ability to infect a wide range of hosts; including domestic ruminants (cattle, buffaloes, goat and sheep), non-ruminant animal species (pigs, dogs, horses, cat etc.,) free ranging animals (Blue bulls, rabbits) and sub-human primates (Chiodini *et al*., 1984; Hermon-Taylor *et al*., 2000). The association of MAP with Crohn's Disease (CD) in human beings has been under intense investigations and evidences are growing in favor of the role of MAP in causation of CD (Chamberlin and Naser, 2006; Singh *et al*., 2008a; Pierce, 2009). Though, MAP is reported to be endemic in domestic livestock population (Singh *et al*., 2008b; Sharma *et al*., 2008; Kumar *et al*., 2007a) of the country however, information in wild ruminants is limited (Kumar *et al*., 2010).

Super survival ability of MAP in adverse environmental conditions and transmission to free ranging wild animals is major hurdle in the control of MAP infection in farm and farmers herds (Greig *et al*., 1999).

Free ranging wild Indian bison (locally known as Gaur or *Bos gourus*) is an endangered wild life species of Southern India and is widely distributed throughout mainland of South and South-East Asia and Sri Lanka. Global population of wild bison is estimated between 13,000 and 30,000 with around 1000 animals in India.

However, the population of wild bison in India has declined sharply and are now limited to southern part of the country in the Western Ghats and their outflanking hills constituting one of the most extensive strongholds of Indian bison with good numbers in Wynaad-Nagarahole-Mudumalai-Bandipur complex (Ranjitsinh, 1997). Wild Indian bison are herbivores in nature and share the habitat, pastures, environment, water resources and diseases (such as rinderpest and Foot and Mouth disease) with domestic ruminants and other wild animal species. Previous molecular epidemiological studies reported inter-species transmission or sharing of MAP strains by domestic livestock, wild animals and human beings (Pavlik *et al*., 1995, 1999; Singh *et al*., 2009a). Despite high prevalence of MAP in domestic livestock, wild animals and human beings in North India (Singh *et al*., 2008a, b; Kumar *et al*., 2007a), status of MAP infection and the role of wild Indian bison in transmission and genetic evolution of MAP strains has

Corresponding Author: S.V. Singh, Veterinary Microbiology Laboratory, Animal Health Division,
Central Institute for Research on Goats, Makhdoom, PO-FARAH, District Mathura (UP), India

not been investigated in the country so far. Present pilot study aimed to recover and genotype MAP isolates recovered from wild Indian bison population of South India using microscopic examination, culture, IS900 PCR and IS1311 PCR-REA.

MATERIALS AND METHODS

Collection of samples: A total of 13 fecal samples were collected 2 times at 1 year (January 2008 and November 2009) interval from wild Indian bison in the Kodai kanal region of South India. In the upper Kodai hills (part of Nilgiri hills) there is Southern Regional Center of Central Sheep and Wool Research Institute (CSWRI) at Mananvanur near Kodai kanal (Tamil Nadu). The region extends over an area of 1000 km^2 and highest peak measures 2030 m above sea level and at 77-78° longitude and 10-11°N latitude. Total local annual rain fall in the region is recorded as 1055 mm and temperatures range from minimum of 6.6°C to the maximum of 29.9°C. MAP infection has been endemic this sheep flock located at SRC, Mannavanur (Kumar *et al*., 2007b; Singh *et al*., 2009b) and in the domestic livestock population in North India. Wild bison frequently come down from the thick forest in search of food (share grazing resources) and water (water bodies) during the night hours.

Processing of samples: Fecal samples were concentrated by centrifugation and stained with Ziehl-Neelsen (ZN) stain. MAP cultures were isolated as per the method of Whipple *et al.* (1991) with some modifications (Singh *et al*., 1996). Briefly, approximately 2 g of fecal sample was finely grounded in sterilized pestle and mortar in 10-12 mL of distilled water. Ground material (15 mL) was centrifuged at 1557×g for 1 h at Room Temperature (RT), supernatant was discarded and from middle layer smear was made and was decontaminated in 25 mL of 0.9% Hexadecyl Pyridinium Chloride (HPC) for 18-24 h at room temperature. After decontamination and sedimentation, the supernatant was discarded slowly and from ≈1 mL of sediment, slants of Herrold's Egg Yolk Medium (HEYM) with and without mycobactin J were inoculated and smears were also prepared. Slants were incubated at 37°C and screened for the appearance of colonies every 15 days.

IS900 PCR (colony PCR): Cultures were processed for DNA isolation as per the method of Singh *et al.* (2007a) and subjected to PCR using IS900 primers (Vary *et al*., 1990). The 229 bp fragment targeting specific IS900 sequence was amplified from template DNA. Briefly, in a volume of 50 μL of reaction mixture, 1 μL of each primer (forward primer: 150 C 24-mer; reverse primer: 921, 25-mer), 22 μL red dye Master Mix (Taq DNA polymerase, dNTPs, reaction buffer with 1.5 nM magnesium chloride), 24 μL de-ionized water and 2 μL of template DNA were included (total volume 50 μL). A total of 35 cycles was performed in a thermocycler (MJ Research) for complete amplification reaction. The total time taken for 36 cycles was 1.20 h.

The reaction conditions were: initial de-naturation at 94°C for 4 min (1 cycle), de-naturation at 94°C for 10 sec, annealing at 61°C for 10 sec, extension at 72°C for 10 sec (35 cycles) and a final extension at 72°C for 10 min. The presence and yield of the specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis. Positive (MAP Indian Bison type) and negative (sterile liquipure water) controls were also run to check for contamination.

IS1311 PCR: IS1311 PCR was carried out using M56 and M119 primers as per Sevilla *et al.* (2005) with some modifications. Briefly, PCR were set up in volume of 25 μL, using 0.5-1.0 ng template DNA, 2.5 mL of 10×PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs and 1 U Taq (Promega). Thermal cycling was as follows: initial denaturation at 94°C for 3 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplicon sizes of 608 bp were considered positive in IS1311 PCR after separation on 2% agarose gel stained with ethidium bromide.

IS1311 PCR-REA: IS1311 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 IS1311 PCR product, 3 μL 10×buffer (Fermentas) and 2 U of each endonuclease HinfI and MseI (Fermentas). The reaction mixture was incubated at 37°C for 1.5 h. Band patterns were visualized after electrophoresis on 4% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted as per Whittington *et al.* (2001).

RESULTS AND DISCUSSION

Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of Johne's Disease (JD) has emerged as major animal pathogen with significant zoonotic concerns worldwide. Free ranging animal species may play vital role in transmission, evolution and control of MAP infections including JD. Therefore, knowledge of status and genotypes of MAP in wild animals is pre-requisite to restrict the disease in animals and formulate the effective control program to minimize the human exposure to MAP

through food chain. Indian Bison Type genotype of MAP has been frequently (88.0%) reported from the domestic livestock population of the country (Singh *et al*., 2009a; Sharma *et al*., 2008; Yadav *et al*., 2008) however, information in wild ruminants is limited (Kumar *et al*., 2010). Though Sohal *et al*. (2010) tried to trace the evolution of Indian Bison Type genotype of MAP from *M. avium* and Bison Type genotype, first time reported from Montana region of US. Present pilot study first time reported the presence of MAP in wild Indian bison (*Bos gourus*). Prevalence of MAP in wild Indian bison was lower using microscopic examination (46.1%) as compared to fecal culture (61.8%). High prevalence of MAP in wild Indian bison of Kodaikanal region may be due to due to the gregarious nature (family group of wild Indian bison consists 2-40 individuals) and sharing of pasture, grazing area and water bodies with domestic livestock population wherein MAP infection was endemic (Kumar *et al*., 2007b; Singh *et al*., 2009b).

Results of present study are in agreement with the study of Kumar *et al*. (2007a) who reported culture as more sensitive than microscopic examination for the screening of MAP infection in domestic livestock. All the culture isolates (8) recovered were characterized as MAP using IS900 PCR.

Limited studies on genotyping of MAP revealed that Indian Bison type genotype was exclusively recovered from wild Indian bison population in South India (Fig. 1). Recently, Singh *et al*. (2009a) reported that Indian Bison type as the major genotype in domestic livestock, wild ruminants and human population in India. Presences of Indian Bison type genotype of MAP in wild Indian bison extended the list of natural host range of this genotype in the country.

The predominance of MAP Indian Bison Type in cattle, buffaloes, goats, sheep, deer, wild bison, showed ability of this genotype to infect different ruminant species (interspecies sharing) including human beings. More extensive studies on presence and evolution of Indian Bison Type genotype in Indian Bison and its adaptation to other livestock species may provide important link with it's parent genotype, Bison Type which is only limited to wild bison in Montana region of US. The huge population (about 485 million) of domestic ruminants in India where majority of animals are endemically infected with MAP may have important role in the genetic evolution and subsequent adoption in the native bison population.

This Indian Bison Type genotype has been found to be highly pathogenic in the domestic ruminants and is used to make ELISA kits and indigenous vaccine against paratuberculosis in India. Though it has also been

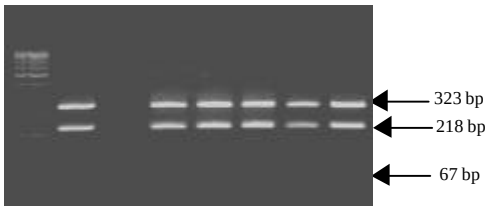

Fig. 1: IS1311 PCR-REA analysis results representative set of MAP isolates; Lane 1 = 1 kb (100 bp) DNA ladder; Lane 2 = Positive control (Indian Bison Type); Lane 3 = Negative control (Distilled Water); Lane 4 = WB2; Lane 5 = WB4; Lane 6 = WB7; Lane 7 = WB11 and Lane 8 = WB13

reported from deer, blue bulls, monkey and human beings but its pathogenicity to these species has not been studied so far. Diagnostic kits and vaccine based on Indian Bison type (Singh *et al*., 2009b; 2007b) has been extensively used by the researchers for the diagnosis and control (therapeutic vaccine) of MAP infection in domestic ruminants in India.

CONCLUSION

Wild Indian bison is an endangered livestock species in India. Government has been running conservation programs in national parks at Karnataka (Nagarhole and Bandipur) and Assam (Kaziranga and Manas) to increase their numbers.

Through, MAP infection cause negative energy balance and reduce the reproductive performance of the host (Hasonova and Pavlik, 2006) therefore, more epidemiological studies are required to estimate true prevalence of MAP infection and JD control programs should be in place to determine the role of bison with respect to MAP (as reservoir of infection or in transmission) and conserve this endangered species in India.

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