Comparative Immunology, Microbiology and Infectious Diseases 34 (2011) 163–169

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Molecular epidemiology of *Mycobacterium avium* subspecies *paratuberculosis*: *IS900* PCR identification and *IS1311* polymorphism analysis from ruminants in the Punjab region of India

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ARTICLE INFO

Article history:
Received 23 February 2010
Accepted 28 September 2010

Keywords:
Johne's disease
Mycobacterium avium subspecies *paratuberculosis*
Ziehl–Neelsen staining
PCR
IS900
IS1311
REA
Molecular epidemiology

ABSTRACT

Johne's disease is chronic granulomatous infectious enteritis of animals caused by *Mycobacterium avium* subspecies *paratuberculosis*. A total of 153 animals from 19 dairy farms, 2 gaushalas (unproductive-animal rehabilitation centers), 2 goat and 2 sheep farms from different districts of the Punjab region were selected on the basis of clinical signs of disease. All samples from cattle ($n = 86$), buffalo ($n = 34$), goat ($n = 25$) and sheep ($n = 26$) were subjected to Ziehl–Neelsen staining and DNA extraction by a freeze and thaw method. Ziehl–Neelsen staining detected 71% samples positive for acid-fast bacilli whereas *IS900* PCR detected 55% positive for Map DNA. *IS1311* PCR-REA analysis of *IS900* positive samples revealed 'Bison' type as the most prevalent (82%) genotype of Map, infecting all domestic ruminants. 'Cattle' type was present in a minority of cases (15%) from cattle, buffaloes and goats. This is the first report of 'Cattle' type Map from buffalo and goat species in India.

1. Introduction

Livestock contributes a substantial part to a country's economy in form of milk, meat and draught power. India has a large population of cattle (176.59 million), buffalo (98.7 million), goats (125.46 million) and a huge reservoir of sheep (64.27 million) [1]. Despite this, per animal production is low. One of the major factors responsible for uneconomical returns from the livestock sector is the presence of chronic diseases like bovine viral diarrhea, paratuberculosis, and tuberculosis. Paratuberculosis or Johne's disease (JD); caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map) is characterized by chronic granulomatous enteritis and is present worldwide. The zoonotic implications of Map in Crohn's disease [2] and milk as potential source of Map [3] has emphasized the need of rapid and sensitive detection of the bacterium from animals. Estimated losses of $200–250 million annually to the US dairy industry were due to JD [4]. Though responsible for high morbidity, decreased production, and increased culling, the impact of the disease has not been estimated in India. However, Map has been frequently reported in cattle, buffalo, sheep and goat of northern India [5–7].

The test and cull strategy of JD control programs require sensitive and specific diagnostic techniques. Fecal culture is considered as the gold standard for the diagnosis of Map

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doi:10.1016/j.cimid.2010.09.002

infected animals but requires 12–16 weeks [8] therefore alternative serological and molecular techniques have been employed for diagnosis. The *IS900* element is an insertion sequence considered to be a Map-specific gene with 15–20 copies per genome and is a target for rapid detection of Map by PCR [9]. Direct PCR contributes not only in reducing the time of diagnosis but also in detecting potentially uncultivable Map strains. So far no direct fecal PCR test has sufficient simplicity and proven analytical sensitivity and specificity to be of practical benefit. Understanding of the heterogeneity of Map is important in diagnosis, epidemiological investigation and in designing control measures. Though *IS900* RFLP analysis and genome restriction on pulsed field gel electrophoresis were widely used typing methods to differentiate Map into 'sheep-type' (S) or type I, 'cattle-type' (C) or type II, and 'intermediate' or type III isolates but these methods require large quantities of good quality DNA, which is a difficult proposition in the case of extremely slow growing type I/III isolates. Marsh et al. [10] developed PCR-restriction enzyme analysis (PCR-REA) based on *IS1311* polymorphisms. *IS1311* is, an insertion sequence present in 17 copies in the Map genome and 7–10 copies in *M. avium* subspecies *avium* (Maa). *IS1311* REA, being an easy and rapid technique was used to distinguish between Maa and both cattle and sheep strains of Map based on C/T polymorphism at base position 223. A new band pattern using *IS1311* REA was discovered in bison isolates from Montana (USA) [11]. The reports of interspecies transmission of Map [12] have emphasized the need of genotyping studies in identifying the origin of transmission and preferential host or geographical adaptation of Map strains.

The present study was aimed at standardizing a direct fecal PCR for identification of Map and *IS1311* PCR-REA genotyping of Map prevalent among different ruminant species in the Punjab region of India. The study was also extended to estimate JD status at gaushalas; non-profit organizations running in rural and urban India for the care and protection of nonproductive, culled cattle.

2. Materials and methods

2.1. Collection and processing of fecal samples for DNA extraction

Animals were screened on the basis of clinical signs of chronic diarrhea or weakness. Fecal samples from cattle ($n = 86$) and buffalo ($n = 34$) from 19 dairy farms and 2 gaushalas (unproductive-cattle rehabilitation centers) in the Ludhiana, Barnala, Jalandhar districts and Chandigarh (Punjab) were collected. Goat ($n = 25$) and sheep ($n = 26$) fecal samples were collected from four herds in the Ludhiana region. Feces were concentrated by resuspending in 8–10 ml distilled water followed by centrifugation at 2500 rpm for 45 min at room temperature. After discarding the supernatant, smear was prepared from interface layer for Ziehl–Neelsen (ZN) staining. The overnight decontamination of interface layer [13] using 0.9% hexadecyl pyridinium chloride was followed by DNA extraction as per Garrido et al. [14] with some modifications. The fecal sediment was washed four times with PBS and resuspended in 50 μl of distilled water. The resulting mixture was subjected to heating at 95 °C for 20 min followed by rapid chilling in ice for 20 min. Four cycles of such freeze and thaw were followed by centrifugation at 8000 rpm for 5 min. The resulting supernatant was used as template in the PCR reaction.

2.2. IS900 PCR

IS900 PCR was performed as per Marsh et al. [10] using P90B and P91B primers with some modifications. Briefly, in a total 50 μl reaction mixture, 38 μl red dye master mix, 1 μl each primer (10 pmole/μl) and10 μl DNA template was used. Thermal cycling was performed in a MJ research thermal cycler (GMI, Minnesota, USA) and cycling conditions were as follows, initial denaturation at 94 °C for 3 min, followed by 37 cycles for denaturation at 94 °C for 30 s, annealing of primers at 64 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The 'candidate strain' positive control DNA was provided by the Microbiology Laboratory, Central Institute for Research on Goats (CIRG, Makhdoom, India). The presence and yield of specific PCR product (413 bp) was analyzed by agarose gel electrophoresis.

2.3. IS1311 PCR-REA

Samples positive for *IS900* PCR were processed for *IS1311* PCR using M56 and M119 primers [10]. The reaction mixture composition and amplification conditions were the same as for *IS900* PCR except that an annealing temperature of 62 °C was used. A DNA band size of 608 bp was considered positive for *IS1311*. The *IS1311* REA was performed as per Sevilla et al. [15]. The 40 μl of *IS1311* PCR product was digested for 1.5 h at 37 °C with 10 μl restriction enzyme mixture containing 0.25 μl of 10 U/μl HinfI, 0.25 μl of 10 U/μl MseI (Fermentas), 4.5 μl of distilled water and 5 μl of 10X buffer. Band patterns were interpreted on 4% agarose gel electrophoresis as indicated by Whittington et al. [11] according to which; Map sheep strain type were defined by two bands of 285 and 323 bp (all *IS1311* copies have C at 223 bp); Map Cattle strain type were characterized by four bands of 67, 218, 285 and 323 bp (some copies with a C and some with a T); Map Bison strains were identified by 3 bands of 67, 218 and 323 bp (T in all copies) and 3 bands of 134, 189 and 285 bp were characterized as Maa (C at 223 and T at 422 bp).

3. Results

3.1. ZN staining

Screening of 153 fecal samples by ZN staining detected 110 (71.89%) animals positive for acid fast bacilli, of which 54 (79.4%), 24 (70.5%), 15 (57.6%) and 17 (68%) fecal samples belonged to cattle, buffalo, sheep and goat respectively. All 13 samples (100%) from culled cattle of gaushalas and 65 samples (73.03%) from organized dairy farms were positive for acid-fast bacilli. Of 110 ZN positive samples, 64 (58.18%), 31 (28.18%), 12 (10.9%), 3 (2.7%) were categorized