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Non-chemical method of DNA recovery and characterization of *Mycobacterium avium* subspecies paratuberculosis using IS 900 PCR

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In the present study, two methods of DNA isolation-routine, traditional and standard DNA isolation protocol for Mycobacteria (Method 1) and a new non-chemicals and non-enzymes (physical) method (Method 2) of DNA recovery have been compared and evaluated in IS900 PCR for the specific detection of pathogen. Using the new Method 2, DNA has been recovered from few (1 – 3 colonies), extremely minute and stunted colonies. DNA, thus, isolated from these colonies (colonies PCR) and cultured for the first time from the cases of Crohn's disease in human beings, dairy cattle, raw milk and pasteurized commercial milk samples has been characterized in the present study. It is the first report from India.

Keywords: DNA, Johne's disease, *Mycobacterium avium* subspecies paratuberculosis, PCR

Johne's disease (JD) caused by *Mycobacterium avium* subspecies paratuberculosis (MAP), is a serious infection of animals specially ruminants and also responsible for Crohn's disease, a human infection[1-3], worldwide. Despite high priority, Johne's disease continues to cause huge losses (200 million USD per year) to US cattle industry[4]. However, in India Johne's is not a priority, despite low per animal productivity. Information on prevalence and production losses cause by JD in 465.50 million domestic ruminants has neither been estimated nor realized, mainly due to lack of diagnostic kits and reagents and control measures. Diagnosis of JD is difficult due to long incubation, absence of characteristic symptoms, problems in cultivation of MAP and non-specific results in Johnin test, resulting from similarity of MAP and *Mycobacterium avium* genomes. The two genomes are >98% similar and ribosome rRNA sequence is the same for both [5]. Culture ('Gold Standard' test) employs differential requirements of an iron chelator (mycobactin) as medium supplement for *in vitro* growth of MAP in comparison to other Mycobacteria that are able to synthesize mycobactin. *In vitro* growth of MAP is characterized by extreme fastidious nature and mycobactin J dependency. Till 1989, when IS900[6] was discovered in MAP genome, amplification in PCR for the characterization of MAP was difficult. IS 900 is specifically present in 14-18 copies[6,7] in MAP and absent from other Mycobacteria, thereby facilitating specific identification of MAP in PCR.

Despite 16 years of IS900 discovery, IS900 PCR is still not very convenient test to standardize for characterization of MAP, because of difficulties encountered in isolation of genomic DNA. MAP has rigid cell wall and is very difficult to break thus, isolation of MAP DNA is extremely difficult[8,9]. Pathogenic Mycobacteria, including MAP, contain additional capsular structure-CAP, consisting of sugars, lipo-arabinate and lipo-polysaccharides bound to fatty acid radicals in the electron-translucent membrane of the cell wall. Such a structure provides stability to cell wall as well as poor permeability for hydrophilic and hydrophobic compounds including many antibiotics[10]. This structure inhibits DNA detection therefore, MAP cells must be primarily prepared before DNA isolation, resulting in laborious and not much reliable DNA isolation protocols. For other microbes, methods of DNA releasing are relatively easier and cells do not require initial processing. Prior to enzymatic method of DNA isolation from these hardy Mycobacteria, French press

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method was employed to burst the cell wall and release genetic material[11].

Selection of an adequate method of isolation of mycobacterial DNA is essential and largely determines the success of PCR test in MAP. Comparative studies by Wards *et al.*[12] are beneficial in deciding the adequate DNA isolation methods. The best method for isolation of mycobacterial DNA is combination of physical, chemical and enzymatic methods[13,14] and successfully employed by van Soolingen *et al.*[15], van Embeden *et al.*[16] and Kumar *et al.*[17]. These methods however, require loopful of culture as starting material. But in the recent past, samples from different animals species and sources were cultured for isolation of MAP to study the molecular epidemiology, the colonies were extremely minute and slow growing which were many times lost on prolonged incubation due to contamination or drying of media and failed to increase in size and number. Therefore, the desired loopful of growth was never available for characterization of these colonies by PCR. Since many of these colonies were first time cultured from human and pasteurized milk in the country, therefore molecular characterization was essential. Attempts to isolate DNA from these few colonies and subsequent testing in PCR were consistently negative. Fastidious nature of MAP from sheep and human sources[1,18,19] and special requirements of bovine isolates[20] are well known. Aging of growth promoter may be another reason for stunting of MAP colonies on artificial media. Therefore, aim of this study was to compare the routinely used standard DNA isolation method with non-chemical (physical) method for DNA isolation and subsequent characterization of DNA from MAP colonies by IS900 PCR (colony PCR).

Materials and Methods

A total of 139 cultures; one to few colonies characterized on the basis of slow growth, mycobactin J dependency and acid-fast nature isolated from tissues of animals, tissues and stool samples of human beings, un-pasteurized milk, commercial pasteurized milk and fecal samples of dairy cattle were considered primarily as MAP colonies. The MAP colonies obtained from different species and samples were extremely minute and fewer in number (1-4) and rarely growth was luxuriant. Of 139 cultures, 70 were subjected to freeze and thaw process (Method 2) and the rest of 69 cultures were subjected to DNA isolation protocols of van Soolingen *et al.*[15] and van Embeden *et al.*[16] (Method 1) most commonly used for isolation of Mycobacterial DNA. There was homogeneity in the samples processed by two methods in terms of colonies size and numbers (Table 1).

Preparation of *M. paratuberculosis* template DNA from culture

Method 1—Of the 139 cultures, DNA was isolated by the van Soolingen *et al.*[15] and van Embeden *et al.*[16] methods in 69 cultures.

Method 2—The 70 cultures were subjected to DNA isolation using new freeze and thaw method. MAP growth ranging from single to pauci-bacillary on Harrold's Egg Yolk Medium (HEYM) were harvested in 100 µl of triple distilled water or normal saline. The suspension was subjected to heating at 90°C in water bath for 10 min, followed by snap cooling in ice for 10 min. Heating and cooling steps were repeated 3 times. The Eppendorfs were then centrifuged at 2432 × *g* for 5 min and supernatant was used directly as template DNA for IS900 PCR amplification using specific primers.

Estimation of DNA quantity—Standard Pico-green analysis was used to determine the DNA concentration in culture samples processed by Method 1 and Method 2.

DNA amplification—Template DNA prepared by Method 1 and Method 2 were subjected to IS 900 PCR using Vary primers[7]. PCR reaction mix (50 µl) contained 0.2 m*M* each of 4 dNTPs, 1 µ*M* primers, 1.5 m*M* $MgCl_2$, 1U of Taq polymerase (Qiagen), and template DNA (1.0 µl) isolated by Method 1 and Method 2. The Q-solution was used in PCR after optimization in our laboratory as recommended by the manufacturer. PCR temperature conditions were—94°C for 4 min; 30 cycles at 94°C for 10 sec, 61°C for 10 sec, 72°C for 10 sec and final extension at 72°C for 10 min. PCR products were separated in agarose gel (2%) at 5 V/cm for 30 min, stained with ethidium bromide and visualized (Gel Doc, Alpha Innotech).

Table 1—Number of colonies per culture processed by two methods

Colonies	Method 1	Method 2
1	10	10
2	15	23
3	29	28
4	15	9
Total	69	70

Results

Recovery and estimation of DNA concentration— Using DNA isolation by Method 1; 23 cultures (33.3%) yielded DNA and DNA concentration ranged from 13.86-18.54 ng/µl (Table 2). By Method 1; DNA was isolated from the cultures containing 4 and more visible colonies and from 10 samples containing 3 visible colonies whereas, using Method 2, DNA was recovered from all the 70 cultures and concentration of DNA ranged from 3.0 to 10.0 ng/µl (Table 2).

Characterization of MAP DNA by IS900 PCR— Sixty three (90%) cultures were characterized as MAP, of the 70 cultures processed for DNA isolation by Method 2 and subsequent amplification by specific IS900 PCR. Of the DNA obtained from 16 human, 13 pasteurized milk, 7 un-pasteurized milk and 34 faecal cultures and amplification in IS900 PCR, confirmed, 14 (87.5%), 13 (100%), 7 (100%) and 29 (85.3%) cultures, respectively (Table 3). While of 69 cultures processed by Method 1, DNA was obtained in 23 (33.3%) samples and 10 (14.5%) were characterized as MAP by IS900 PCR (Table 3).

Discussion

MAP is hardy, slow-growing, Gram-positive and acid-fast bacilli[21,22]. MAP can be differentiated phenotypically from *M. avium* and *M. sylvaticum* by its dependence on mycobactin[23] and geno-typically by the presence of multiple copies of an insertion element, IS900[6,24]. Restriction endonuclease analysis has identified variations in 2 principal types of MAP, a cattle type (C) and a sheep (S), by Collins *et al.*[25]. This information is not available about the Indian MAP isolates[26] from different geographical regions and species. Therefore, the diagnosis of Johne's disease depends on detection of MAP in clinical samples and in view of the difficulties in isolation of either MAP in culture or MAP DNA in PCR, not much work has been undertaken on JD and MAP. Optimization of method of isolation of genomic DNA from MAP is difficult, therefore lot of variability has been observed in the results of different workers. Culture of MAP is still considered the 'Gold standard' for diagnosis of JD. However in routine cultures, growth of primary MAP colonies was extremely slow and rarely luxuriant. This may be due to variations in animal species investigated. Sheep strains are known to be more fastidious[19] than MAP from other animal species. Quality and quantity of MAP culture obtained was directly dependent on condition of growth promoter, Mycobactin J (personal communication). MAP from different animal species show variation and has diverse requirements of medium components. Sodium pyruvate is added for culture of bovine MAP isolates[20]. During large-scale culture of MAP from different species (goats, sheep, cattle, buffaloes, blue bulls and human beings) using faeces/stool, milk (pasteurized and un-pasteurized) and tissues, these difficulties have been encountered[27]. This has led to non-availability of loopful of MAP

Table 2—Estimation of the quantity of DNA obtained by the two methods

Colonies	Concentration of total DNA recovered (ng/µl)	
	Method 1	Method 2
1	-	3.0-4.0
2	-	3.5-5.4
3	-	5.0- 8.0
4	13.86-18.54	8.0-10.0

Table 3—Comparative results of IS900 PCR of cultures processed by Method 1 and Method 2 for DNA isolation

Samples	Source (place)	No of samples	DNA Recovery	IS900 PCR Positives
Method 1				
Milk (Un-pasteurized)	Cattle (Ludhiana)	45	15 (33.3)	3 (6.6)
Feces	Blue bulls (Farah)	9	2 (22.2)	1 (11.1)
Tissues	Goats (Farah)	15	6 (40.0)	6 (40.0)
Total		69	23 (33.3)	10 (14.5)
Method 2 (New method)				
Milk (Un-pasteurized)	Cattle & buffalo (Farah & Mathura)	13	13 (100.0)	13 (100)
Milk (Pasteurized)	Market (Mathura, Agra, New Delhi)	7	7 (100.0)	7 (100)
Faeces	Cattle, (Mathura)	34	30 (88.2)	29 (85.3)
Tissues/Stools				
Human CD patient (New Delhi) & in contact persons (Farah)				
Human		16	14 (87.5)	14 (87.5)
Total		70	64 (91.4)	63 (90.0)

Figures in parenthesis denote per cent

colonies/growth for DNA isolation and subsequent confirmation by IS900 PCR and also restricting genotyping of MAP cultures by PCR-REA and genomic studies. Therefore, employing method 1 for the isolation of DNA[15,16] from deficient quantities of MAP cultures (less than optimum-loopful) as recommended in the protocol was counter productive. Using this method MAP cultures could be characterized only from multi-bacillary cultures, which were obtained in very few cases from tissues of goats and feces of blue bulls. In species such as sheep it is the paucibacillary form of MAP that predominates and prolonged incubation period up to years may be required to grow the organism[18]. Large number of MAP colonies which were minute and never grew in size despite prolonged incubation were lost most of the times due to drying of HEY medium or contamination, therefore such MAP cultures from human beings, pasteurization milk and cattle could not be characterized for long time. During isolation of DNA from few colonies the success rate was poor due to lowered availability of Mycobacterial cells per sample. Isolation of DNA from these little tiny colonies was also difficult due to loss of DNA in the multi-step DNA isolation method 1. This is a practical difficulty, which has been responsible for variability in sensitivity of PCR test by different workers[28,29,30].

Low sensitivity of the PCR reaction for the detection of MAP in tissues, faeces, milk and culture samples is attributed to the false negative results due to difficulties involved in the preparatory phase (bacteria concentration, DNA extraction and purification, presence of few bacilli and presence of PCR amplification inhibitors, which often led to false negative results. PCR is unable to differentiate viable and killed MAP as both may be present in pasteurized milk. A rapid, sensitive and specific method to detect viable MAP in milk or other dairy products is important for monitoring and to check human risk. PCR test targetting specific IS900 gene sequences is a promising alternative for specific detection by isolation of MAP from different clinical samples within short time.

Cell wall of MAP is difficult to breakdown, which causes additional problem in DNA isolation procedure. There is not a single recommended uniform rapid and efficient protocol available for DNA extraction from MAP despite several methods described for Mycobacterial species and requiring loopful culture growth[11,15,16,31,32]. However, in the present study, *a new method* was adopted wherein DNA was successfully isolated even from a single tiny colony of MAP, without using any chemical, enzyme and hectic multi-step process of DNA isolation, which increased the sensitivity of PCR manifolds. Therefore, it helped to overcome the low sensitivity of PCR assay (*method 1*), due to low yield of DNA from tiny MAP colonies. It was presumed that repeated freeze and thaw method lysed sufficient proportions of MAP cells, thereby allowing the DNA release and detection by PCR. Method 1 yielded DNA only from samples containing more than 3-4 colonies and the method 2 of DNA isolation, recovered DNA from every sample and concentration of DNA ranged from 3 to 10 ng/µl. This concentration of DNA was sufficient and detected MAP in IS900 PCR. In routine testing 2-5.0 ng/µl DNA concentration has been standardized in PCR in our laboratory (personal communication). *Method 2* would be very promising to detect viable MAP in pasteurized milk and can save MAP isolates for other purposes that have few colonies even after prolonged incubation. Comparative results indicated better sensitivity of *method 2* over *method 1* (multi-step DNA isolation process). Success of PCR amplification depended on the quality and quantity of the used as template DNA. Sensitivity of PCR was poor in detecting DNA from minute colonies of MAP. Method 1 was applied unsuccessfully in extraction of sufficient amount of DNA from pauci-bacillary cultures. In the present study *method 2* was applied for DNA isolation from tiny MAP colonies however, this method may be employed directly on clinical samples (faeces, milk, tissues and blood) with certain modifications (removal of PCR inhibitors present in clinical samples).

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