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Review Article

Mycobacterium avium subspecies paratuberculosis diagnosis and strain typing—Present status and future developments

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Mycobacterium avium subspecies *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic gastroenteritis of ruminants and has zoonotic importance. We present here a review of MAP with respect to—(i) present diagnostic techniques and important developments; and (ii) MAP strain-typing tools. A summary of the findings to date is presented, and advantages and disadvantages of each of the methods are compared and discussed.

Keywords: Antigens, *Mycobacterium avium* subspecies paratuberculosis, Specific genetic markers.

Paratuberculosis (Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a fatal inflammatory bowel disease of ruminants characterized by long pre-clinical phase where chemotherapy is ineffective and uneconomical. Paratuberculosis is listed B disease (OIE) and has trade restrictions. Disease is usually underreported due to difficulties in diagnosing pre-clinical cases. Malabsorption of nutrients leads to negative energy balance in the body and is protein loosing entero-pathy of domestic ruminants[1]. Clinical paratuberculosis results in chronic intermittent diarrhoea, which is non-responsive to antibiotics and / or anthelminitic treatment, therefore leading to progressive wasting, emaciation and death. MAP has wide host range including domestic and wild ruminants, free grazing animals, birds, farm animals and also human beings. Paratuberculosis has high economic impact on animal industry, where premature culling, reduced carcass value, reduced weight gain, increased susceptibility to other infections, reduced fertility, reduced feed efficiency, reduced milk quality and quantity and additional veterinary costs resulting in estimated annual loss of USD 250 million in US alone[2, 3]. Besides, the disease is costly to agriculture industry also has public health concerns (zoonotic). Though paratuberculosis is endemic in India[4-12], but economic losses have never been estimated or realized.

Effective control of paratuberculosis has been hampered due to lack of rapid and accurate diagnostic tests. Test results from the sub-clinically infected animals are a challenge to interpret, because clinical signs are not present to assist their interpretation[13]. Transmission of infection usually takes place prior the tests becoming positive and before clinical signs are developed. Other important issue is the lack of discriminatory power of widely used strain typing techniques. Other problem that limits the MAP typing is extremely fastidious nature and difficulties in primary isolation of this hardy bacillus. The data are insufficient to guide disease control programs. Only 2 kinds of MAP strains C type (isolates of cattle, goat other animals and human beings) and S type (sheep isolates) have been described in detail. We summarize present knowledge and important developments in MAP diagnostic and typing techniques in the present study.

MAP diagnostic approaches—Critical issues that affect paratuberculosis diagnosis are prolonged incubation period and the fact that non-lethal tests

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must be used. Diagnostic tests to detect MAP infection can be divided into 2 categories, one that detects the organism (bacteriology and molecular approaches) and other that detects host reactions to the organism (immunological approaches).

I. Bacteriology

i. Direct microscopy—Staining of fecal samples for acid-fast bacilli (AFB) may reveal mycobacterial bacilli, but the sensitivity of this method is low. Moreover, accurately distinguishing MAP from non-pathogenic mycobacteria (saprophytes) in such samples can be difficult even for experienced persons, resulting in low specificity.

ii. Conventional culture—Isolation of MAP by culture is Gold Standard test (100% specific)[14]. Clinical samples including, feces, tissues (intestine, MLN, SMLN, liver, testes, udder, uterus, etc), blood and milk can be used to isolate MAP from infected animals. Culture medium either HEYM (Herrold's Egg Yolk Media) or modified LJ (Lowenstein-Jensen) supplemented with iron chelator (mycobactin) are preferred by diagnostic laboratories to isolate MAP[15,16]. MAP colonies are characterized on the basis of slow growth (appearing after 8 weeks), mycobactin J dependency, acid-fastness and IS900 PCR. Culture can detect infected animals shedding more than 100 CFU/g of feces[17] and sensitivity of fecal culture is 50%[18]. The disadvantages of culture methods are—(a) long incubation period (2 months to years); (b) lack of reproducibility; (c) MAP from sheep and human beings frequently fail to grow; and (d) MAP is not distributed homogenously in feces, it is highly desirable that fecal samples collected from MAP infected animals may not contain MAP.

iii. Radiometric culture (BACTEC)—This method is adapted from the one used to isolate *M. tuberculosis* in humans. Collins *et al*.[19] have demonstrated that BACTEC system, if modified, could also be used to diagnose paratuberculosis. The main advantage of this method over the standard one is that it can detect low numbers of MAP and can detect the bacterium faster (in 7 weeks). Disadvantages are that the BACTEC method is more expensive, requires specialized instrument and involves handling of radioisotopes.

II. Immunological tests

i. Cell mediated immunity (CMI)

Skin testing or delayed type hypersensitivity (DTH)—This test is performed by intradermal inoculation Johnin (extract of MAP). An increase in the thickness of the skin at site of injection >4 mm within 24-72 hr is considered as positive. It is less sensitive (54%) and specific (79%), also has poor correlation with the infectious status of the animal[20].

Interferon-gamma detection (IFN-γ)—IFN-γ is released by lymphocytes of infected animals after stimulation with antigens. Two assays known as bioassay[21] and sandwich enzyme immunoassay (EIA) have been evaluated[22]. However, results indicated that the test is non-specific and interpretations are uncertain. Thus, IFN-γ detection has limited value in diagnosing paratuberculosis[23, 24].

ii. Humoral immune response

AGID—The AGID test has a high specificity (> 90%) in animals with clinical signs, but because of poor sensitivity (30%)[20] this test is not popular.

Complement fixation test (CFT)—The specificity of CFT is less than AGID and ELISA. Moreover, this test detects antibodies 1 to 5 months later than ELISA[25] and has intermediate sensitivity to AGID and ELISA. Results yield false positives and false negatives, CFT is not recommended as routine diagnostic test.

ELISA—ELISA is most widely used 'Herd-screening test' for diagnosis of paratuberculosis. Sensitivity of ELISA is high in clinically infected animals and is low at initial and terminal stages of disease (Anergy). In general, ELISA sensitivity for clinical and sub-clinical cases is 85 and 15%, respectively[20]. Absorption of serum with *M. phlei* removes non-specific antibodies[14]. An indigenous ELISA kit utilizing protoplasmic antigen from native MAP 'Bison type' strain has been developed for screening of animals and also human beings[8, 9, 26].

Interpretation of the results of serological tests should be made with caution, since evaluation of performance (sensitivity and specificity) of serological results is done by comparing with culture (Gold standard) and many times poor standardization or over sensitivity of culture may lead to wrong estimates of the sensitivity and specificity of serological test. To avoid errors in the interpretation of ELISA results, S/P ratio (sample to positive ratio) method has been proposed[8, 27] that relates ELISA OD values with the disease status of infected animal.

III. Molecular diagnosis

DNA probe (non-culture detection)—Insertion sequence IS900, discovered in the late 1980s[28], is most widely used genetic marker for specific detection of

MAP. DNA probes based on IS900 enable detection of MAP without cultivating bacteria, hence are faster (in less than three days). DNA probes are costly and difficult to perform.

PCR—IS900 PCR is the most widely used molecular test to diagnose MAP infection. When applied to confirm MAP colonies IS900 PCR has 100% specificity and sensitivity. PCR assays have also been optimized to screen clinical samples (tissues, blood, milk and feces) [5-12]. However, PCR is less sensitive than culture when applied to screen clinical samples due to presence of inhibitory substances or non-recovery of DNA. Another concern is non-specificity of IS900 due to presence of IS900 like sequences in non-MAP mycobacteria[29].

IV. Infection stage and sensitivity of tests

Performance of particular diagnostic test depends on stage of disease. DTH tests and intestinal cultures are usually positive before fecal culture and serology or clinical signs. Table 1 represents the infection stage and performance of particular test in that stage. High sensitivity for serological tests and fecal culture is only possible when animals are in clinical stage of disease. Usual mix of animals (sub-clinical, clinical and advance clinical) in a herd renders herd-level sensitivity of ELISA about 45%[30] and faecal culture about 45-55%[31]. According to some workers these sensitivity estimates are too high and the actual figure for both fecal culture and ELISA in cattle may be about 35%[32]. However, results of studies by Singh *et al*.[4-7] showed that the estimates of positives in ELISA kit are always low as compared to culture (double or three times) in goats, sheep, cattle and buffaloes, using milk, feces, tissues, vaginal swabs, serum and milk in culture, PCR and ELISA test. In terminal stages due to immune anergy[33, 34] sensitivity of serological tests may be as low as 10-25%[35,36]. Thus, detection of paratuberculosis in a herd at a single point of time with a single diagnostic test is difficult.

V. Serology and fecal culture

The observations indicate that fecal shedding commences sooner than the detectable humoral response. Results of longitudinal studies confirm that fecal shedding of MAP starts before 6-9 months of the sero-conversion[37, 38]. A minority of animals does seroconvert before faecal shedding and animals can be faecal culture negative, but ELISA positive[39]. Again it's difficult to be certain about the validity of comparison between culture and serological tests since sensitivity of culture method varies with method used and the results are not reproducible. Moreover, the organism is unevenly distributed within the faeces, contributing to false negative culture outcomes or the perception that shedding is intermittent. Notwithstanding the problems mentioned above, fecal culture is more sensitive overall in detection of sub-clinical infection.

VI. Fecal culture and tissue culture

Culture of target tissues (intestine and MLN), is more sensitive than fecal culture. Animals negative in successive fecal cultures may have positive intestinal tissue culture. This occurs because paucibacillary cases are likely to be detected only by culture of tissues. Multibacillary cases shed greater numbers of organisms, explaining the greater efficacy of fecal culture in their detection. Culture of biopsies of intestinal tissues and associated lymph nodes is a sensitive diagnostic approach in cattle, but is seldom used. For sheep and goat it has only been used experimentally. As the data is limited for goat and sheep, biopsy needs to be evaluated in parallel with serology and fecal culture before it can be recommended in this species.

VII. Testing strategy for paratuberculosis

In large population sizes testing of every animal in a herd is impractical and uneconomical. Rapid and cost effective tests like ELISA should be used, as 'herd screening test'. ELISA positives can be tested by culture or PCR. ELISA can be repeated at 3-4 monthly intervals to monitor the titers.

Table 1—Performance of diagnostic tests at particular disease stage

Test	Disease stage	Specificity	Sensitivity
DTH and IFNγ	Silent and Sub-clinical	Moderate	Moderate to high
Culture of intestine	Silent, Sub-clinical and Clinical	High	High
Culture of feces	Sub-clinical and Clinical	High	Moderate to high
Serology	Sub-clinical and Clinical	Moderate to high	High
Molecular tests	Sub-clinical and Clinical	High	Moderate

VIII. New antigen discoveries and MAP diagnosis

Pre-absorption of sera with *M. phlei* is a good option for increasing assay specificity, but reduces the sensitivity. Reports indicate that only 1/3rd of animals shedding MAP can be detected by present ELISA tests[27]. It is essential to characterize promising antigens for sero-diagnosis. The search for MAP specific antigens has led to discovery of many antigens as follows—

i. 34 kDa cell surface protein[40, 41]— Carboxy terminal end is 100% specific for MAP.

ii. Secreted antigens (9, 14 and 34 kDa)[42]—All are recognized by sera of clinically and sub-clinically infected animals.

iii. Ahp C and Ahp D[43]—Both have ability to differentiate paratuberculosis and tuberculosis.

iv. p43[44]—Product of IS900, specific antigen for MAP diagnosis.

v. 14kDa secreted antigen[45]—Higher levels of antibodies against this antigen were detected in patients with Crohn's disease.

vi. 35kDa antigen[46, 47]—In ELISA, this antigen has highest sensitivity compared to other antigens (Ag85 A, B, C and SOD).

With complete genome sequencing of international reference isolate MAP K10 post genomic applications will play a pivotal role in identifying MAP specific antigens and some work has already been started in this area.

(i) Results of comparative genomics and immunoproteomic analysis of MAP extracts and culture filtrates 25 MAP diagnostic antigens were identified[48].

(ii) *In silico* comparison of MAP genome with other mycobacterial genomes and cloning and expression of MAP unique protein sequences MAP specific protein antigens were identified that reacted with sera from infected animals[49].

(iii) Comparative genomics and immunoblots identified14 antigen including Mod D, Pep A, Arg J, Cob T, Ag85C and 9 hypothetical proteins[50], but the sero-diagnostic evaluation of these antigens reveal that natural forms of these proteins are more useful for sero-diagnosis, since recombinant proteins have lower sensitivity to crude antigens[51].

(iv) Comparative genomic approach[52] identified 21 potential coding sequences specific for MAP and found that 5 of these sequences code for MAP specific antigens[53].

Newly identified molecular targets for MAP diagnosis—Genome sequencing of MAP K10 had identified 3 more MAP unique IS elements; ISMav2, ISMAP02 and ISMAP04 present in 3, 6 and 4 copies, respectively in MAP K10. Small-scale studies using these newly identified MAP unique IS elements have provided encouraging results. Studies have shown that PCR for ISMav2, ISMAP02 has comparable sensitivity and specificity to IS900 PCR for detection of MAP in fecal samples and are 100% specific for MAP[54-56]. Comparative genomics based study had identified 17 MAP unique large sequence polymorphisms (LSPs)[57] and PCR analysis for these LSPs across the 383 *M. avium* complex isolates (107 MAP +276 non-MAP) reveals that LSP^P2, LSP^P4, and LSP^P15 are 100% specific for MAP, others are also present in non-MAP mycobacteria. Of these MAP specific LSP^Ps, LSP^P15 consistently give positive results (highly sensitivity) across MAP isolates and are heterogeneously distributed among different MAP isolates.

MAP strain typing tools

Genetic analysis of pathogenic bacteria is important since traits such as host range and virulence are non-randomly distributed among phylogenic lineages. Molecular epidemiological research has long been hampered due to difficulties in culturing MAP and few numbers of MAP isolates have been maintained in available collections. Despite of hurdles past two decades has seen increased interest and application of molecular strain typing methods for identifying genetic diversity within MAP. The recent sequencing international reference isolate MAP K10 genome has helped to identify additional molecular markers for better differentiation of MAP isolates. We here summarize the current state of MAP strain typing techniques.

Phenotypes, biochemical typing and serotyping

Two phenotypes of MAP isolates have been described based on the pigmentation and rate at which they grow[58]. One phenotype is pigmented (yellow), extremely slow growing requires minimum 16 weeks of incubation usually reported from sheep (type 1). Colonies of the other phenotype are non-pigmented and appear significantly faster (6 to 12 weeks) usually reported from cattle goat and the other animals. Human MAP isolates are cell wall deficient (spheroplasts) and may require years to emerge in the form of colonies. The slow growth characteristic of MAP is a

major limitation to these phenotypic methods. Other methods including serology, biochemical assays[59], gas-liquid chromatography[60], and antimicrobial susceptibility[59] not capable of differentiating MAP isolates.

i. IS900 RFLP—Collins *et al*.[61] have first used IS900 RFLP, to differentiate MAP isolates and subsequently, Pavlik *et al*.[62] have proposed a standardized IS900 based RFLP. However, IS900 RFLP has lower discriminatory index, only able to divide MAP isolates into two distinct groups, one seen predominantly in cattle, goats other animals and human beings (C type), while the second observed in sheep[62-64]. RFLP has yielded excellent results in mycobacteria[65], but its use for MAP typing is limited since the method is time consuming, labour intensive and requires large quantities of high quality DNA and suffers with lower discriminatory power. Extreme slow growing nature of MAP further limits the use of RFLP based approaches.

ii. IS1311 RFLP and PCR-REA—IS1311 RFLP also divides MAP isolates into the same groups as IS900 RFLP[66, 67]. This also suffers with disadvantages of lower discriminatory power and others as described for IS900 RFLP. A PCR-restriction endonuclease analysis (REA) method that targets a point mutation in IS1311 sequences at 223rd and is able to divide of MAP isolates into 3 groups: cattle, sheep and bison[68,69]. Bison type genotype has been initially observed from MAP isolates of Montana, USA and later this genotype is observed in Indian MAP isolates[70, 71, 6-8, 11] from different host species (buffalo, cattle, goat and sheep). Although IS1311 is not unique to MAP, IS1311 PCR-REA has the advantage over IS900 RFLP in terms of simplicity of the test and higher discrimination power; moreover, it can be applied directly on clinical samples to target the strain diversity of unculturable MAP strains (mostly from sheep and humans).

iii. Pulse field gel electrophoresis (PFGE)—Some studies have revealed that PFGE is unable to distinguish between MAP isolates from different hosts and diverse geographical locations[72, 73, 74]. Others have reported that PFGE is able to divide MAP isolates in two main groups, cattle and sheep type[58, 71, 75].

iv. Multiplex PCR for IS900 Loci (MPIL)—This method is based on IS900 locus polymorphism in different MAP isolates (Presence/Absence of IS900 at defined locus), and involves a multiplex PCR for different IS900 loci. Studies indicate[76, 77] that like IS900 RFLP and IS1311 RFLP, MPIL also divides MAP strains into 2 major groups (cattle and sheep type). Despite some technical advantages over RFLP, MPIL does not provide any additional discrimination and hence have limited application in strain typing of MAP.

v. Amplified fragment length polymorphism (AFLP)—AFLP is more discriminatory than above-mentioned methods[77] and divides MAP isolates into 3 major clusters. First cluster consisting of isolates from bovine, goats and mouse, second cluster consisting of isolates from sheep and third cluster consists of isolates from human origin and other unknown hosts, indicating a clear segregation. Although AFLP to have a greater resolving power than MPIL and RFLP, it suffers from the limitation that the allelic variation is indexed at anonymous biallelic sites or locations. Additionally, repeatability and interlaboratory variation limits its application in multiple site analyses[78].

vi. Variable number tandem repeat (VNTR) analysis—Overduin *et al*.[79] and Biet *et al*.[80] have employed 5 and 8 polymorphic VNTR loci to type MAP isolates and able to generate 6 and 12 VNTR types, respectively. Both the studies are able to subdivide predominant IS900 RFLP type (R01) type into various subtypes. Thus, VNTR typing is a convenient tool for epidemiological surveys. But, the data on the use of VNTR typing for studying MAP diversity is limited.

vii. Short sequence repeat (SSR) typing—Genotyping techniques described as above have their limitations or have not been evaluated adequately on large number of isolates. It is the SSR typing that has generated epidemiological meaningful data. Amonsin *et al.*[81] have initially used SSR typing based on 11 SSR loci for the typing of the MAP isolates and observed 3 clades, one consisting of cattle isolate, second consisting of goat isolates and third consisting of sheep isolates, they observed that greater diversity is observed among cattle, followed by goat and then sheep isolates. They showed that goat isolates have more similarity to cattle than for sheep isolates. SSR typing studies have successfully shown that certain SSR types (genotypes) are associated with subclinical disease and others are associated with clinically overt disease[77, 82-84]. SSR analysis has also helped to identify genotypes that are either host restricted or shared among the isolates (inter-species transmission). Ghadiali *et al.*[83] have shown that 52% of the total isolates (domestic animals, wild animals, humans) were containing 7 G and 4 GGT genotype

(7g4ggt) for G and GGT repeat SSR loci, revealing that it is the most predominant and successful genotype in terms of the virulence. This 7g4ggt genotype has also been reported from MAP isolates of blue bulls, cattle, buffaloes, sheep and goats (Sohal, J. S., unpublished data). Transcriptional analysis of the macrophages exposed to MAP isolates showed that for common SSR types specific signaling pathway is observed regardless of the host of origin[84].

viii. Single nucleotide polymorphisms (SNPs) and MAP typing—Study on SNP markers for MAP isolates is limited and there is only one study till date. Marsh *et al*.[85] have studied SNPs using PCR and sequencing based comparison of S and C MAP strains at 30 loci across 29 MAP genes. They found 8 genes contained a total of 11 SNPs in S strain compared to C strain. Sequences from both the strains are used to query the MAP K10 genome. The C strain is identical and 11 SNPs are confirmed in S strain.

Strain sharing between humans and animals

Molecular analysis of human and animal origin MAP isolates reflects strong sharing of isolates. Francois *et al*.[86] have applied IS900-RFLP to assess the degree of similarity between MAP isolates from animals with paratuberculosis and humans with CD. The results of this analysis reveal the restricted genetic heterogeneity among isolates collected from CD patients and the major group of animal isolates. Other studies based on IS900 RFLP yielded similar results[63]. The results from the other typing techniques (MIRU-VNTR typing) have also shown that MAP isolates from humans (CD patients) cluster with animal isolates[79, 80]. Extensive study on the analysis of short sequence repeats (SSRs) of MAP isolated from CD patients by Ghadiali *et al.*[83] who have identified two alleles, both of which clustered with strains derived from animals with paratuberculosis.

Close similarity of the human isolates with isolates derived from animal species is suggestive of inter- and intra-species transmission and an association of MAP isolates with the pathobiology of Crohn's disease (zoonotic potential). Alternative to this close association in molecular epidemiological studies is indicative of common ancestry of human and animal MAP isolates. But, the whole genomic comparisons, at transcriptome and proteome levels, of isolates derived from human and animal hosts may yield additional insight into a variation in biological function and/or host adaptation.

Concluding remarks

Accurate diagnosis of infected animals is prerequisite of disease control programs. Efforts are needed on the development of simple, rapid and non-invasive tests based on the newly identified antigens. Range of diagnostic tests is available, but all have inborne limitations. Culture and molecular tests are very specific, but are less sensitive and costly. For molecular tests, it is important to evaluate the newly identified molecular markers on large number of samples since concern of IS900 specificity for MAP has been raised. But consistent with mobile nature of newly identified MAP specific markers (IS elements and LSPs), they may be variably present among different MAP isolates. Thus, a diagnostic test targeting different markers (LSPs and IS elements) can be designed in multiplex form to test particular clinical sample or MAP colony. Among all tests, ELISA is most affordable and widely used, but it also demands improvements in terms of sensitivity and specificity. Improvement of ELISA requires identification of infection stage and MAP specific antigens to improve the assay sensitivity and specificity. All newly identified antigens may impart major impact on development of diagnostics for paratuberculosis.

Another important tool that can guide the disease control program is epidemiological observations. The knowledge of genetic diversity of the pathogen is important in terms of their virulence, antibiotic susceptibility and host preferences. A plethora of molecular tests is available for assessing genetic diversity of MAP. New molecular approaches with higher resolution power (SSR analysis, VNTR analysis, SNP typing etc.) can be used to study diversity and molecular phylogeny.

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