Current diagnostic approaches for Paratuberculosis- A Review

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Abstract

Diagnosis of chronic bacterial diseases like paratuberculosis is a major challenge in dairy herds. ELISA for the detection of Mycobacterium avium subsp. paratuberculosis (MAP) specific antibodies and direct fecal PCR for detection of MAP are regularly used for diagnosis of subclinical infections and are recommended for demonstration of the population or individual animal freedom from MAP infection. For confirmation of clinical cases culture, histopathology and PCR are recommended. For estimation of prevalence/surveillance, ELISA is recommended. The relative merits and shortcomings of the available methods for paratuberculosis diagnosis are reviewed in the context of purpose of testing.

Key words: Paratuberculosis; diagnosis; ELISA; Fecal PCR; culture; fitness of purpose.
Introduction

Paratuberculosis (Johne’s disease) is a chronic incurable granulomatous enteritis of ruminants caused by Mycobacterium avium subsp. paratuberculosis (MAP). Calves are at the highest risk of infection with MAP during their first weeks of life and fecal-oral route is the primary route of transmission. The disease is known for its unusually long incubation period and prolonged clinical course with no symptoms in the early and subclinical stage but with the risk of intermittent fecal shedding of bacteria. The first clinical signs of paratuberculosis are usually not seen until animals are 2-6 year old (Clarke, 1997). Johne’s disease is characterized by protein losing enteropathy, manifested as intermittent diarrhoea and progressive cachexia. Emaciation/ weight loss, decreased milk production, anaemia, infertility and intermandibularedema (bottle jaw), are the prominent signs during clinical stage of the disease. Infected animals excrete MAP mainly by feces. MAP infection is incurable and control by treatment of sick animals is neither practically feasible nor cost effective. Cattle with clinical disease having symptoms like chronic diarrhoea and bottle jaw die eventually.

Johne’s disease (JD) is now worldwide in distribution due to the global trade of animals, incurring huge economic losses to the dairy industry. The disease has wide host range and affects many ruminant species like cattle, sheep, goats, deer, llamas, bison etc. (Harris and Barletta, 2001). Herd prevalence of JD in the US dairy herds is around 68 per cent (Kirkpatrick et al., 2011) and it is 20 per cent among cattle in several countries of Europe (Nielsen and Toft, 2009). Significantly high prevalence of MAP infection has been reported in sheep and goat (22.5%), and in many organized cattle and buffalo herds (29%) in India (Kumar et al., 2007; Singh et al., 2008; Narnaware and Tripathi., 2017). JD is a contributing factor for the poor per animal productivity of Indian livestock and restrictions on animal trade.

Control of paratuberculosis depends on population-level measures such as identification and culling of animals that are shedding MAP, applying hygienic measures to prevent exposure of neonatal/young calves with the dung of adult cattle.

Diagnosis of Paratuberculosis

The diagnosis of paratuberculosis is aimed at the diagnosis of clinical disease and the detection of subclinical infection. The latter is a highly challenging task but is crucial for effective control of the disease. The detection of subclinical infections is based on indirectly measuring the MAP specific antibodies in serum or the demonstration of cell-mediated responses or by direct detection of MAP in the faeces or tissues by culture or PCR. Clinical cases of JD are confirmed by the demonstration of MAP in the faeces by culture and the polymerase chain reaction. The same methods along with histopathology can be used for diagnosis at necropsy for demonstrating MAP in tissues.

Purpose of testing

Animals or herds may be tested for different purposes. Validity of any paratuberculosis diagnostic test should be considered in the context of the purpose of testing. The world organization for animal health, OIE advocates the concept of ‘fitness of purpose’ in validation of diagnostic tests, and testing is undertaken for the following purposes: (1) to demonstrate population ‘freedom from infection’ (Zero prevalence); (2) to demonstrate freedom from infection in individual animals for trade purpose; (3) to aid in eradication programmes; (4) to confirm diagnosis of clinical cases; (5) to estimate prevalence of infection (surveillance); (6) to determine immune status in individual animals or populations post vaccination. Test accuracy estimates are considered to be valid only for the purpose for which the test has been validated (Gardner et al., 2011).

Fecal PCR and ELISA are recommended for demonstration of the population freedom from MAP infection and for individual animal freedom from infection (OIE, 2020). Whereas, for confirmation of clinical cases culture, histopathology and PCR are recommended. For estimation of prevalence/ surveillance, ELISA is recommended. Both ELISA and culture often misdiagnose animals in the early/ subclinical stages of the disease (Magombbedze et al., 2017). The use of indirect tests that measure host’s cell-mediated immune response to MAP infection viz. IFN-gamma test and delayed type hypersensitivity (DTH) test, are only limited to checking the immune status post vaccination and are of less value in diagnosis.

Cell-mediated immune responses (CMI) with IFN-γ secretion predominate in early stage of infection and later shifts to Th2 response characterized by antibody production when the disease progresses. Hence, animals with clinical disease tend to lose Th1/ IFN-γ response. But, in a proportion of animals with clinical signs of JD, both Th1/ IFN-γ and Th2/ antibody responses may be seen (Begget et al., 2011; Stabelet et al., 2015). A commercial IFN-γ test kit (Bovigam, USA) developed for bovine tuberculosis diagnosis is being used for detection of early/subclinical infections but this kit has not been validated by the manufacturer for paratuberculosis diagnosis. Moreover, the specificity and sensitivity of the test varies greatly depending on the interpretation criteria (Kaliset al., 2003; Nielsen &Toft, 2008). The Johne skin test gives false positive results as sensitisation to the M.avium complex is widespread in animals and interpretation of results is complicated by the lack of agreement in interpretation criteria (OIE, 2020). Both the tests are not recommended in JD diagnosis (OIE, 2020; Collins, 2011; Collins et al., 2006).
Table 1: Recommended diagnostic tests for Paratuberculosis

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Commercial dairy cattle</th>
<th>Commercial goats</th>
<th>Commercial sheep</th>
<th>All seed stock/ breeding stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control programme in MAP infected high prevalence (&gt;5% test - positive) herds</td>
<td>ELISA</td>
<td>ELISA</td>
<td>Pooled fecal culture or PCR</td>
<td>Fecal culture, or PCR on individual animal</td>
</tr>
<tr>
<td>Surveillance</td>
<td>Environmental or pooled fecal culture</td>
<td>Environmental or pooled fecal culture</td>
<td>Environmental or pooled fecal culture</td>
<td>Not recommended</td>
</tr>
<tr>
<td>Eradication</td>
<td>Pooled fecal culture or pooled fecal PCR</td>
<td>Pooled fecal culture or pooled fecal PCR</td>
<td>Pooled fecal PCR</td>
<td>Fecal culture, or PCR on individual animal</td>
</tr>
<tr>
<td>Confirm a clinical diagnosis on animals in herds with no prior confirmed MAP infections</td>
<td>Necropsy, fecal culture, or PCR on the affected animal</td>
<td>Necropsy, fecal culture, or PCR on the affected animal</td>
<td>Necropsy or fecal PCR on the affected animal</td>
<td>Culture or PCR and histopathology on biopsy or necropsy-collected tissues</td>
</tr>
<tr>
<td>Confirm a clinical diagnosis on animals in herds proved to be MAP infected</td>
<td>ELISA, fecal culture, or PCR</td>
<td>ELISA, fecal culture, or PCR</td>
<td>Fecal PCR</td>
<td>Culture or PCR and histopathology on biopsy or necropsy-collected tissues</td>
</tr>
</tbody>
</table>

(Source of data in this table is from Collins et al., 2006 and Collins MT, 2011.)

MAP testing regulations for international trade/movement of livestock

The world organization for animal health (OIE) offers no recommendations on movement requirements for paratuberculosis. The International Association for Paratuberculosis (IAP) therefore provided guidelines for exporting and importing countries for movement of livestock, consistent with the principles of sanitary and Phytosanitary Agreement of World Trade Organization (Kennedy et al., 2017). As per IAP, fecal PCR, fecal bacteriological culture and indirect antibody ELISA are appropriate to detect MAP infection and certification as ‘MAP infected’. OIE provides an overview of various tests for MAP diagnosis for different purposes (Refer Table 1 Test methods available for diagnosis of paratuberculosis and their purpose, OIE Terrestrial Manual chapter 2.1.11 Paratuberculosis 2020).

Diagnostic tests have low sensitivity until later stages of infection and hence a negative test of individual animal has a low predictive value. Continuous herd or population testing and active surveillance on a large scale over years is required to demonstrate freedom from infection (Kennedy et al., 2017). Surveillance and formal control programs for Paratuberculosis are present in Australia, United States of America and most of the developed countries in North America and Europe (Whittington et al., 2019). Such countries prefer to import animals for breeding only from MAP Certified herds and will enact quarantine procedures that are sufficient to manage the risk of MAP infection while importing livestock from countries like India (Kennedy et al., 2017).

Fecal Culture

Fecal culture is the gold standard method for diagnosis of Johne’s disease, but culturing MAP is labor intensive, has low sensitivity and can take up to 16 weeks or even longer because of fastidious nature of the organism, the slow progression of the disease, intermittent and low shedding of MAP in faeces. Extraction of DNA difficult and PCR inhibitors in the faeces inhibit the amplification of DNA in PCR.

Fecal PCR

For many years, PCR detection of MAP in faeces has become the most reliable diagnostic tool because it is possible to identify subclinical and clinical animals that are shedding MAP. PCR has superior sensitivity in comparison to all other test methods, and specificity comparable to fecal culture (Giese and Ahrens, 2000). The efficiency of direct PCR is influenced by the DNA extraction method. The thick waxy MAP cell wall makes the extraction of DNA difficult and PCR inhibitors in the faeces inhibit the amplification of DNA in PCR.
(Stevenson and Sharp, 1997) and can lead to false negative results (Whipple et al., 1992). Clinical specimens usually have very few MAP. Several commercial fecal DNA extraction kits have been evaluated for their use in MAP diagnosis. Reports suggest that the DNA extraction kits give varied levels of sensitivity of the MAP DNA recovery from samples, According to Leite et al. (2013) the kit (ZR Fecal DNA Miniprep, Zymo Research Corp., Irvine, CA) employing physical disruption (bead beating) of the sample in a lysis solution is found to be ideal for isolating MAP DNA from feces with 94.1% sensitivity. The QIAmp Stool DNA Minikit, QuiagenNc has the sensitivity of 53% as it fails to detect low(<10 CFU) and moderate shedders (10-100 CFU of MAP/sample).

PCR based on IS900, a 1451 bp insertion sequence specific for MAP(McFadden et al., 1987) is routinely employed. Being present in 14-18 copies in MAP genome it offers higher sensitivity of PCR methods (Vary et al., 1990). The IS900 PCR has better sensitivity than standard culture techniques (Vary et al., 1990; Giese and Ahrens, 2000). However, presence of IS 900 like elements is reported in some mycobacterial species other than MAP (Cousins et al., 1999; Tasara and Stephen, 2005), hence confirmation for making culling decisions should be substantiated by culturing. The other gene targets unique to MAP and used for rapid identification by PCR are ISMav2, F57, and ISMap02 sequences (Stabel&Bannantine, 2005; Strommenger et al., 2001; Vansnick et al., 2004). The IS 900 PCR and F57 PCR methods that are developed by Vansnick et al. (2004) are widely in use for MAP diagnosis as they offer high sensitivity and specificity. The restriction enzyme analysis of an insertion sequence IS1311, common to MAP and M. avium subsp. avium, and MAP 1506- sequence analysis can be used to distinguish between these species and for typing of ovine, bovine and bison strains of MAP (Sevilla et al., 2005; Whittington et al., 1998; Griffiths et al., 2008; Chaitanya et al., 2015).

Real time PCR
In recent years, real-time PCR methods have been developed to detect MAP from different specimens viz. blood, milk, faeces, tissues and environmental samples. Again, the quality of nucleic acid sample greatly influences the efficiency of real time PCR. Therefore, a DNA extraction method that ensures maximum MAP DNA recovery and high quality of DNA is a critical step to use in real-time PCR (Parka et al., 2014). IS 900 specific Real time PCR methods offer greater sensitivity than bacterial culture (Fang et al., 2002; Schonenbrucher et al., 2008) and can detect very low numbers (less than 10 CFU, Mahony and Hill, 2004; Khare et al., 2004; Nelli et al., 2008) of MAP. A high throughput direct fecal PCR assay that involves recovery of MAP bacteria from fecal suspension and, rupture and DNA extraction by magnetic bead beating performed in tandem with IS 900 quantitative PCR is developed and approved for use in JD control programmes in Australia and New Zealand (Plain et al., 2014). However, accurate quantification of MAP organisms present in the sample is not possible with IS 900 gene target because of its presence in variable copy numbers. The MAP specific single copy gene, F57 is targeted for absolute quantification of MAP in the specimens (Tasara and Stephen, 2005; Irenge et al., 2009; Chaitanya et al., 2019).

ELISA for MAP specific antibodies:
The ELISA for serum antibodies to MAP in cattle is most commonly used for all the OIE stated testing purposes except for the confirmatory diagnosis of clinical cases (OIE, 2020). ELISAs are useful in estimating prevalence with in herds. MAP specific antibody titres are detectable later than CMI and correlate with severity of lesions and sensitivity of ELISA is low in animals shedding low numbers of MAP (Whitlock et al., 2000) and non fecal shedding animals (Dargatz et al., 2004). The sensitivities and specificities of ELISA vary greatly because the time from occurrence of antibodies to fecal shedding varies (Nielson & Toft, 2008). The commercial ELISA kits involve pre-absorption of test sera with soluble M. phlei antigen prior to testing in an indirect ELISA are used in diagnosis. Pre-absorption step eliminates nonspecific cross-reacting antibodies (Molina et al., 1991). In infected populations, a much higher number of animals are expected to react in tests for CMI compared with antibody tests, as CMI is indicative of exposure while antibodies indicate progress of infection (OIE, 2020). Commercial ELISA kits imported from abroad viz. Paracheck (Prionics AZ, Switzerland) and HerdCheck (IDEXX, USA) are used for Paratuberculosis diagnosis in cattle and goats in India. In sheep, the antibody response is more frequently detected in multibacillary than in the paucibacillary form of the disease. ELISA is less useful in sheep than in cattle and goats. For sheep, PCR remains as the only effective diagnostic tool.

Testing of replacement animals by ELISA is strongly advocated prior to purchase and only ELISA negative animals should be purchased (Collins, 2011). If an animal is positive in ELISA, confirmatory test, based on detection of MAP by culture or PCR should be done for definitive evidence of MAP infection. Periodic screening of herds to detect subclinical infection is essential to check the prevalence of the infection and control of the disease by the disposal of positive reactors. The same approach is also followed to check freedom from infection, and thus to identify low risk herds for safer trade (Collins et al., 2006; Collins, 2011). USDA-APHIS recommends screening of cattle twice at 6 month interval using ELISA and fecal culture. In the United States pre-purchase testing of the replacement animals is mandatory. Testing of the herd of origin is
much more reliable than testing only the purchased animals. If the infection is established in a herd, separation of calves from cows immediately after birth is recommended along with the elimination of positive reactors to serological tests or faecal shedders.

Over all, the sensitivity of the current diagnostics for Paratuberculosis (detection of MAP in faeces by culture or PCR and by ELISA detection of MAP specific antibodies) is not high enough to reliably detect all animals in the subclinical stage of infection as these animals excrete MAP in low numbers and may not yet develop measurable antibody titre. Diagnostic sensitivity values of PCR may have been hyped due to comparisons of PCR with culture techniques having relatively low analytical sensitivity values. The sensitivity of serologic diagnostic assays is low in the early stages of the disease due to the predominance of cellular immune response with IFN-gamma secretion (Stabel, 2000). One has to select the appropriate diagnostic test for the intended purpose. Multiple herd-level prevalence surveys should be performed in order to identify the positive reactors as no single diagnostic test is able to detect all the animals at different stages of infection. Positive animals must be segregated from the herd promptly.

**Conclusion:** The OIE recommended ELISA for the detection of MAP specific antibodies and direct fecal PCR for detection of MAP for diagnosis of subclinical infections and for demonstration of the population or individual animal freedom from MAP infection. For confirmation of clinical cases culture, histopathology and PCR are recommended. For estimation of prevalence/ surveillance (screening of dairy herds), ELISA is recommended. Johnin test (Delayed Type Hypersensitivity reaction) lacks specificity and is not recommended for Johne’s disease diagnosis. Multiple herd-level prevalence surveys should be performed in order to identify the positive reactors as no single diagnostic test is able to detect all the animals at different stages of infection. USDA-APHIS recommends screening of cattle twice at 6 month interval using ELISA and fecal culture.

**References**


