Current diagnostic approaches for Paratuberculosis- A Review

R.K. Chaitanya*, K. Lakshmi Kavitha and B. Sreedevi

Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati-517502, INDIA.
*Corresponding author:Phone: +919440225858; E-mail-chaitanyaerk@gmail.com

Journal of Livestock Science (ISSN online 2277-6214) 13: 201-207
Received on 1/6/22; Accepted on 26/8/22; Published on 1/9/22
doi. 10.33259/JLivestSci.2022.201-207

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) isa chronicincurable 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 paratuberculosisare 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 andrestrictions 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 histopathologycan 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 paratuberculosisdiagnostic 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).

FecalPCR 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 (Magombedze*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 (Begg*et al*., 2011; Stabel*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 (Kalis*et al*., 2003; Nielsen &Toft, 2008). The Johnin 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

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

(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 Interntional 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 Paratuberculosisare 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'sdisase, but culturing MAP is labour intensive, has low sensitivity and can take up to 16 weeksor even longerbecause of fastidious nature of the organism, the slow progression of the disease, intermittent and low shedding of MAP in faeces by subclinically infected animals. Culture of MAP from faeces or tissues requires double-decontamination of the specimens to eliminate other bacteria and fungal spores by treating with Hexadecylpyridinium chloride and antibiotics (Vancomycin, Amphoterecin B and Nalidixic acid), which result in decrease in the viability of MAP (Reddacliff*et al*., 2003) and low sensitivity (range from 38-55 *per cent*, Whitlock *et al*., 2000) of the culture methods.Primary requirement for Mycobactin J addition in culture media (Randall and Collins, 1992) makes culturing an expensive method. Liquid culture media like Middlebrooks 7H9 allows relatively faster culturing and detection of MAP as few as one to ten organisms per gram of faeces (Pozzato*et al*., 2011) when compared to Herrolds egg yolk medium and Lowenstein Jensen medium. Fluorescence based automated MAP culture system like BACTEC MGIT 960 allows rapid detection of MAP as low as 10 CFU/ml (Shin *et al*., 2007) and is most commonly used in MAP diagnosis at present.

Fecal PCR

For many years, PCR detection of MAP in faeces has becomethe most reliablediagnostic tool because it is possible to identify subclinial 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 feceswith 94.1% sensitivity. The QIAmp Stool DNA Minikit, QuiagenInc 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, a1451 bpinsertion 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 IS*Mav*2, F*57*, and IS*Map*02 sequences (Stabel&Bannantine, 2005; Strommenger*et al*., 2001; Vansnick*et al*., 2004). The IS 900 PCR and F*57* 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 IS*1311*, common to MAPand *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 ensuresa 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 cattleis 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 (Nielsen & 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 anitibodies) is not high enoughto 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

1) Begg DJ, De Silva K, Carter N, Plain KM, Purdie A, Whittington RJ, 2011. Does a Th1 over Th2 dominancy really exist in the early stages of *Mycobacterium avium* subspecies *paratuberculosis* infections? Immunobiology 216**:** 840-846.
2) Chaitanya RK, Reddy YKM,Arthanari T, 2015. Strain typing of *Mycobacterium avium* subsp. *paratuberculosis* from Tamil Nadu, India based on polymorphisms in MAP1506 locus and IS1311 PCR-REA.Advances in Animal and Veterinary Sciences, 3 (5): 289-294.
3) Chaitanya, R.K., Reddy, Y.K.M., Dhinakar Raj, G. and Thangavelu,A. 2019. Quantification of *Mycobacterium avium* subsp. *paratuberculosis*from the tissues of challenged mice using SYBR Green real time PCR assay for the assessment of vaccine efficacy. Indian Journal of Animal Research 53(7): 944-948.
4) Clarke CJ, 1997. The pathology and pathogenesis of paratuberculosis in ruminants and other species. Journal of Comparative Pathology 116: 217-261.
5) Collins MT, 2011. Diagnosis of paratuberculosis.Veterinary Clinics of North America: Food Animal Practice 27: 581-591.
6) Collins MT, Gardner I, Garry FB, Roussel AJ, Wells SJ, 2006. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. Journal of American Veterinary Medical Association 229 (12): 1912-1919.
7) Cousins DV, Whittington R, Marsh A, Masters A, Evans RJ,Kluver P, 1999. Mycobacteria distinct from *Mycobacterium avium* subspecies *paratuberculosis*isolated from faeces of ruminants possess IS 900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis. Molecular and Cellular Probes 14: 431-442.
8) Dargatz DA, Byrum BA, CollinsMT, Goyal SM, HietalaSK, JacobsonRH, Kopral CA, Martin BM, McCluskey BJ, Tiwari D, 2004. A multilaboratory evaluation of a commercial enzyme linked immunosorbent assay test for the detection of a commercial enzyme linked immunosorbent assay test for the detection of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in cattle. Journal of Veterinary DiagnosticInvestigation 16: 509-514.
9) Fang Y, Wu WH, Pepper JL, Larsen JL, Marras SA, Nelson EA, Epperson WB, Christopher-Hennings J,2002. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples.Journal of Clinical Microbiology 40:287-291.
10) Gardner IA, Nielsen SS, Whittington RJ, Collins MT, Bakker D, Harris B, Sreevatsan S, Lombard JE, Sweeney R, Smith DR, Gavalchin J, Eda S, 2011. Consensus based reporting standards for diagnostic test accuracy studies for paratuberculosis in ruminants. Preventive Veterinary Medicine 101: 18-34.
11) Giese SB and Ahrens P,2000. Detection of MAP in milk from clinically affected cows by PCR and culture. Veterinary Microbiology **77**:291-297.

12) Griffiths TA, Rioux K, Buck JD, 2008. Sequence polymorphisms in a surface PPE protein distinguish Types I, II and III of *Mycobacterium avium* subsp. *paratuberculosis*. Jounal of Clinical Microbiology 46: 1207-1212.
13) Harris, NB and Barletta RG,2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine.Clinical Microbiology Reviews 14:489-512.
14) Irenge LM, Walravens K, Govaerts M, Godfroid J, Roseels V, Huygen K, Gala JL, 2009. Development and validation of triplex real-time PCR for rapid detection and specific identification of *M. avium* subsp. *paratuberculosis* in fecal samples.Veterinary Microbiology 136: 166-172.
15) Kalis CH, Collins MT, Hesselink JW,Barkema HW, 2003. Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. Veterinary Microbiology 97: 73–86.
16) Kennedy D, Benedictus G, Nielsen S, Lybeck K, Schwan E, Frossling J, Sergeant E, Kelton D, Nauholz H, 2017. Guidelines for certification with respect to the movement of livestock for *Mycobacterium avium*subsp*paratuberculosis*(MAP) infection. Version 1.3. Paratuberculosis News https://www.paratuberculosisnet/publicationsphp 4: 3-17.
17) Khare S, Ficht TA, Santos RL, Romano J, Ficht AR, Zhang S, Grant IR, Libal M, Hunter D, Adams LG, 2004. Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-time PCR. Journal of Clinical Microbiology 42: 1075-1081.
18) Kirkpatrick B, Shi X, Shook GE, Collins MT, 2011. Whole genome association analysis of susceptibility to paratuberculosis in Holstein cattle. Animal Genetics 42: 149-160.
19) Kumar P, Singh SV, Bhatiya AK,Sevilla I, Singh AV, Whittington RJ, Juste RA, Gupta VK, Singh PK, SohalJS, Vihan VS, 2007. Juvenile Capri-Paratuberculosis (JCP) in India: Incidence and characterization by six diagnostic tests. Small Ruminant Research 73: 45-53.
20) Leite FL, Stokes KD, Robbe-Austerman S, Stabel JR, 2013. Comparison of fecal DNA extraction kits for the detection of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction.Journal of Veterinary Diagnostic Investigation 25: 27–34.
21) Magombedze G, Tinevimbo S, Eda S, Stabel JR, 2017. Inferring biomarkers for *Mycobacterium avium* subspecies *paratuberculosis* infection and disease progression in cattle using experimental data.Nature Scientific Reports, 7:44765/ DOI: 10.1038/srep44765.
22) Mahony JO and Hill C, 2004. Rapid real time PCR assay for detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*in artificially contaminated milk.Applied Environmental Microbiology70: 4561-4568.
23) McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J, 1987. Crohn's disease – isolated mycobacteria are identical to Mycobacterium paratuberculosis, as determined by DNA probes that distinguish between mycobacterial species. Journal of Clinical Microbiology 25: 796–801.
24) Molina A, Morera L,Llanes D, 1991. Enzyme linked immunosorbent assay for detection of antibodies against *Mycobacterium paratuberculosis* in goats. American Journalof Veterinary Research 52: 863-868.
25) Narnaware SD and Tripathi BN, 2017. Seroepidemiology of paratuberculosis in cattle population of organized and unorganized farms of India.Indian Journalof AnimalSciences 87 (1):21-24.
26) NelliRK, Graham E, Dunham SP, Taylor DJ, 2008.Real time PCR identification of *Mycobacterium avium* subsp. *Paratuberculosis*in ovine and bovine tissues.Veterinary Record 163: 422-423.
27) Nielsen SS and Toft N, 2008. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques.Veterinary Microbiology129: 217–235.
28) Nielsen SS and Toft N, 2009. A review of prevalences of paratuberculosis in farmed animals in Europe.Preventive Veterinary Medicine 88: 1-14.
29) Office International des Epizooties (OIE). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2020. Paratuberculosis. Chapter, 2.1.15
30) Parka KT, Allenb AJ,Davisa WC, 2014. Development of a novel DNA extraction method for identification and quantification of *Mycobacterium avium* subsp. *paratuberculosis* from tissue samples by real-time PCR. Journal of Microbiological Methods 99: 58–65.
31) Plain KM, Marsh IB, Waldron AM, Galea F, Whittington AM, Saunders VF, Begg DJ, De Silva K, Purdie AC, Whittington RJ, 2014. High-Throughput direct fecal PCR assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* in sheep and cattle. Journal of clinical microbiology 52 (3): 745-757.
32) Pozzato N, Gwozdz J, Gastaldelli M, Capella K, Ben CD, Stefani E, 2011. Evaluation of rapid and inexpensive liquid culture system for the detection of *Mycobacterium avium* subspecies *paratuberculosis*in bovine faeces. Journal of Microbiological Methods 84: 413-417.
33) Randall SL and Collins MT, 1992. *Mycobacterium avium* subspecies *paratuberculosis:* Factors that influence Mycobactin dependence. Diagnostic Microbiology and Infectious Diseases 15: 239-246.

34) Reddacliff LA, Vadali A, Whittington RJ, 2003.The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium subsp. paratuberculosis* isolated from tissues and faeces. Veterinary Microbiology 95:271-282.
35) Schonenbrucher H, Abdulmawjood A, Failing K, Bulte M,2008. New triplex realtime PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. Applied Environmental Microbiology 74:2751-2758.
36) Sevilla I, Singh SV, Garrido JM, Aduriz G, Rodríguez S, Geijo MV, Whittington RJ, Saunders V, Whitlock RH, Juste RA, 2005. Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions.Review of Scientific Techniques OIE 24:1061-1066.
37) Shin SJ, Han JH, Manning EJB, Collins MT,2007. Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of the BACTEC MGIT 960 system. Journal of Clinical Microbiology 45:1941-1948.
38) Singh SV, Singh AV, Singh R, Sharma S, Shukla N, Misra S, Singh PK, Sohal JS, Kumar H, Patil PK, Misra P, Sandhu KS, 2008. Sero-prevalence of Bovine Johne's disease in buffaloes and cattle population of North India using indigenous ELISA kit based on native *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' genotype of goat origin. Comparative Immunology Microbiology and Infectious Diseases 31: 419-433.
39) Stabel JR, 2000.Transitions in immune responses to *Mycobacterium paratuberculosis*. Veterinary Microbiology **77**: 465-473.
40) Stabel JR andBannantine JP, 2005. Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of Mycobacterium avium subsp. paratuberculosis in fecal samples. Journal of Clinical Microbiology 43 (9): 4744–4750.
41) Stabel JR, Bannantine JP, Hostetter J, 2015. *Mycobacterium avium* subsp. *paratuberculosis* infection, immunology and pathology of livestock.Centre for Agriculture and Biosciences International, USDA 512-537.
42) Stevenson K and Sharp JM, 1997.The contribution of molecular biology to *Mycobacterium avium* subspecies *paratuberculosis* research. Veterinary Journal 153: 269-286.
43) Strommenger B, Stevenson K and Gerlach GF, 2001. Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from *Mycobacterium avium* subspecies *paratuberculosis*. FEMS Microbiology Letters 196(1): 31–37.
44) Tasara T and Stephen R, 2005. Development of an F57 sequence based real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Applied Environmental Microbiology 71: 5957-5968.
45) Vansnick EL, De Rijk OP, Vercammen F, Geysen D, Rigouts L and Portaels F, 2004. Newly developed primers for the detection of *Mycobacterium avium* subsp. *paratuberculosis.* Veterinary Microbiology 100:197-204.
46) Vary PH, Andersen PR, Green J, Taylor JH, Mc-Fadden JJ, 1990. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium avium* subsp. *paratuberculosis* in Johne's disease. Journal of Clinical Microbiology 28: 933-937.
47) Whipple DL, Kapke PA, Andersen PR,1992. Comparison of a commercial DNA probe test and three cultivation procedures for detection of *Mycobacterium paratuberculosis* in bovine faeces. Journal of Veterinary Diagnostic Investigation 4: 23-27.
48) Whitlock RH, Wells SJ, Sweeney RW, Tiem JV, 2000. ELISA and fecal culture of paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Veterinary Microbiology 77: 387-398.
49) Whittington RJ, Marsh I, Choy E and Cousins D, 1998. Polymorphisms in IS *1311*, an insertion sequence common to *Mycobacterium avium and M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. Molecularand Cellular Probes 12: 349-358.
50) Whittington R, Donat K, Weber MF, Kelton D, Nielsen SS, Eisenberg S, Arrigoni N, Juste R, Saez JL, Dhand N, Santi A, Michel A, Barkema H, Kralik P *et al.*, 2019. Control of paratuberculosis: who, why and how. A review of 48 countries. BMC Veterinary Research 15: 198.