DIAGNOSIS OF PARATUBERCULOSIS BY MICROBIOLOGICAL CULTURE IN VETERINARY RESEARCH INSTITUTE FROM 2001 TO 2018

ROSELIZA R.*, KHOO E., MOHAMMAD FHITRI S., NORMAH M.A., SAIFU NAZRI R., SITI NORHANANI R., NORAZARIYAH M.N. AND FAIZAH HANIM M.S.

Veterinary Research Institute, 59, Jalan Sultan Azlan Shah, 31400, Ipoh, Perak, Malaysia.
* *Corresponding author:* roseliza@dvs.gov.my

ABSTRACT. Paratuberculosis (or Johne's disease) is one of the most economically important diseases in the livestock industry. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* and is characterised by a chronic progressively wasting syndrome. To date, no treatment is available for the disease and it invariably leads to the death of the infected animal. At present, microbiology culture is the gold standard diagnostic procedure to diagnose the disease. Both culture and serological methods are difficult and challenging. This paper reports the prevalence of Johne's disease in ruminants based on microbiological culture submitted to Veterinary Research Institute, Ipoh from year 2001 to 2018. Throughout the 17 years, only 168 suspected Johne's infection samples were received for culturing, including faeces and rectal pinch which were inoculated on selective mycobactin egg yolk media. Two positive cases of bovine, in the states of Sabah and Kedah, were recorded in year 2001 and 2018 respectively. Even though most of the microbiological culture were negative for *M. paratuberculosis*, the presence of the disease could not be overlooked. Due to the challenges in microbiological culture for diagnosis of the disease, the use of molecular techniques in combination with suitable serological methods is recommended in order to improve diagnosis of paratuberculosis in the laboratory.

Keywords: paratuberculosis, Johne's disease, ruminants, culture, VRI.

INTRODUCTION

Paratuberculosis, which is also known as Johne's disease is an emerging disease affecting all wild and domestic animals including cattle, goat, camel, buffalo and deer. It is caused by infection from *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is a fastidious acid-fast organism which is also known as a causative agent of Crohn's disease in humans (Erume *et al*., 2001). The disease is contagious, chronic and progressive. It is characterised by late onset of clinical signs such as diarrheoa, weight loss and 'bottle jaw' syndrome which is prominent during the late stage of infection (Tiwari *et al*., 2006). Johne's disease in cattle is typically present with chronic progressive emaciation and persistent diarrheoa. In sheep, goat and alpaca, the predominant clinical signs of Johne's infection includes weight loss and emaciation (Cousins *et al*., 2002). Clinical signs usually take two or more

years to appear after initial infection, in most of the cases. However Johne's infection in deer has been noticed in animals as young as 12 months old (Cousins *et al*., 2002). Johne's disease affects the ruminant livestock industry particularly in terms of welfare, and reduced meat and milk production. Thus, at the end, causes restrictions on trade (Cousins *et al*., 2002).

Primary transmission of the disease is mainly through the faecal-oral route. Vertical transmission from infected dams to the foetus was also reported. An animal may be infected either through ingestion of contaminated water, milk, feed or manure from infected animals (Tiwari *et al*., 2006). Most herds can get infected through introduction of sub-clinically infected animals. In most of the cases, young animals were found to be more susceptible to the infection compared to adults.

The diagnosis of Johne's disease is difficult in animals as it is a silent infection from unapparent carrier animals (stage I and II). MAP can proliferate in the jejunal and ileal mucosa with intermittent shedding where the low number of organisms is below the detection level by microbiological culture and molecular detection methods (Stevenson *et al*., 2010).

The method of diagnosis is by (1) direct microscopic observation of MAP in the faeces using Ziehl-Neelsen acid fast stain on a culture of the pathognomonic lesion in selective Herrold egg yolk (HEY) containg mycobactin which is an essential growth factor for MAP, or (2) DNA-based techniques such as PCR as recommended by the World Organisation for Animal Health (OIE).

The combination of serological tests such as complement fixation test (CFT), agarose gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) along with faecal microbiological culture have also been applied in many countries. This is because most of the listed tests alone are less specific and sensitive to confirm the disease. Serological tests is a suitable choice for disease detection in a herd population and during its clinical stage when the antibody titres to MAP are measureable. Faecal culture is the gold standard method for diagnosis of paratuberculosis (OIE, 2014). However primary isolation of MAP is laborious and time consuming, therefore making it a difficult decision to cull infected animals from their herd in a rapid disease control action (Stabel and Bannantine, 2005). This study reports the isolation of MAP throughout 2001 to 2018 based on the samples submitted to VRI and discusses the challenges in microbiological culture for the diagnosis of Johne's disease in a laboratory.

MATERIALS AND METHOD

Faecal culture of *M. avium* subsp. *paratuberculosis*

MAP was cultured from faecal samples or rectal pinch of suspected Johne's infections sent by regional veterinary laboratories or farmers. Approximately one gram or a pea-sized faecal sample was added to 10 times its volume of freshly prepared 0.1% acriflavine solution. After standing for two minutes at room temperature, the supernatant was removed, leaving coarse faecal particles behind. An equal volume

of 1% sulphuric acid was added, mixed well and incubated at 37 °C for one hour. The mixture was then centrifuged at 3,000 rpm and the supernatant was discarded. The sediment was cultured in duplicate onto HEY supplemented with mycobactin J (2 mg/litre) HEY without mycobactin. HEY containing mycobactin supplementd with antibiotics amphotericin B (50 mg/litre), 2% malachite green, pyruvic acid, beef extract, natrium chloride, glycerine, agar base and fresh eggs that had been treated in 70% alcohol and HEY without mycobactin. The tubes were incubated at 37 °C for at least 8 weeks. Any tube showing growth of rapid growing acid fast bacilli within 7 days of incubation were discarded and reported as negative Johne's. If no growth of suspected MAP colonies was observed after 8 weeks, the result was reported as negative culture. MAP colonies on HEY and HEY with mycobactin initially appeared as pinpoint and tend to remain small when the colonies were numerous on the slope, soft, translucent, non-mucoid and off-white to cream or buff coloured (Seyyedin *et al*., 2008; Eamens *et al*., 2015). Any presumptive MAP colonies will be further identified using Ziehl-Neelsen stain for screening of acid fast bacteria, colony morphology and other available tests including nested polymerase chain reaction (nPCR).

DNA extraction

Genomic DNA was extracted using a simple boiling method. The presumptive MAP colonies were picked and transferred to a sterile Eppendorf tube containing 100 µl distilled water. The samples were mixed and incubated at 95 °C for 10 to 15 minutes. Then, it was centrifuged at 10,000 rpm for 3 minutes. The supernatants were transferred to another sterile Eppendorf tube and were kept at -20 °C until used.

Nested PCR

The primer sequences used for the detection of MAP-specific genetic elements, ISMap02, for nPCR was selected for identification purpose and was performed according to the standard protocols (Stable *et al*., 2005) in a mastercycler Eppendorf Nexus GXS 1.

The primer sequences for initial amplification was 5'-GCACGGTTTTTCGGATA-ACGAG-3' (forward primer) and 5'-TCAACTGCGTCACGGTGTCCTG-3' (reverse primer) which resulted in a 278 bp product. The primers nested within the first set, 5'-GGATAACGAGACCGTGGATGC-3' (forward primer) and 5'-AACCGACGCCGCCAATACG-3' (reverse primer) were used for a second amplification reaction and yielded a 117 bp product.

The reaction mixture consisted of ultrapure distilled water, Bioline MyTaq™ 2× Mix, 0.2 µM primers and 2 µl DNA template. Negative control consisted of reaction mixture containing distilled water. Positive control was 2 µl of genomic DNA extracted from Johne's CFT-killed antigen provided by the Serology Section of VRI.

Samples were run in 25 µl final volume according to the following protocols: 1 cycle at for 5 min and 20 cycles at 94 °C for 45 s, 58 °C for 1 min and 72 °C for 2 min, followed by final extension cycle at 72 °C for 7 min. For the nPCR, the following protocol was used with 1 µl of the amplicon from the

first PCR used as a template for the second amplification: 1 cycle at 94 °C for 5 min and 30 cycles at 94 °C for 45 s, 60 °C for 1 min and 72 °C for 2 min, followed by a final extension cycle at 72 °C for 7 min. The second PCR products and Bioline Hyperladder 100 bp were electrophoresed in a 1.5% Hydragene agarose gel incorporated with 3 µl Biotium GelRed in 1 × tris-borate EDTA buffer at 90 V for 45 minutes. The gels were analysed and captured on Major Science Gel documentation system.

RESULTS AND DISCUSSION

The highest number of suspected cases, 94, was recorded in year 2001. This was followed by 42 cases and 15 cases for the next two consecutive years. From year 2005 until 2018, the number of suspected Johne's infection reduced tremendously, averaging one case per year. In year 2008 and 2009, no case on isolation of MAP was received by VRI for diagnosis (Table 1).

In 2001, a case of MAP was isolated in cattle from Sabah, however no other detail was recorded. Another case, in 2018, was isolated in cattle in Kulim District, Kedah. Most of the suspected Johne's samples sent to VRI had requested for testing without providing full history and information pertaining to the cases.

Faecal culture results on HEY with mycobactin showed small, translucent, soft and off-white colour colonies after 6 weeks of incubation (Figure 1). Acid-fast bacteria were observed in Ziehl-Neelsen staining examined under oil immersion (×100) with a light microscope.

The colonies were PCR positive and expected PCR product with 117bp size were observed, which corresponded with the band size of the positive control (MAP antigen) (Figure 2).

Based on the data, the number of Johne's cases submitted to VRI for diagnosis were very few. However, the low number of cases reported for the 17 years does not

Table 1. The number of suspected cases and positive isolation received by VRI for diagnosis of Johne's Disease from 2001 to 2018.

Year	Number of cases	Number of positive case
2001	94	1
2002	0	0
2003	42	0
2004	15	0
2005	2	0
2006	1	0
2007	1	0
2008	0	0
2009	0	0
2010	2	0
2011	1	0
2012	2	0
2013	1	0
2014	1	0
2015	2	0
2016	3	0
2017	0	0
2018	1	1
Total	168	2

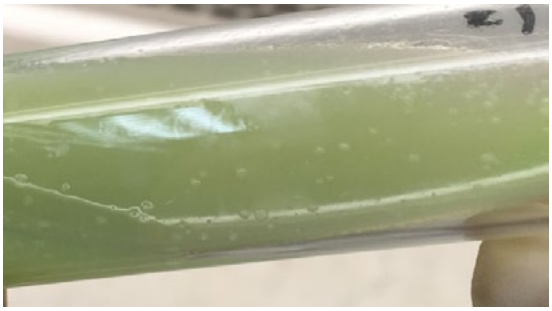

Figure 1. *Mycobacterium avium* subsp. *paratuberculosis* colonies on Herrold's egg yolk medium containing mycobactin J.

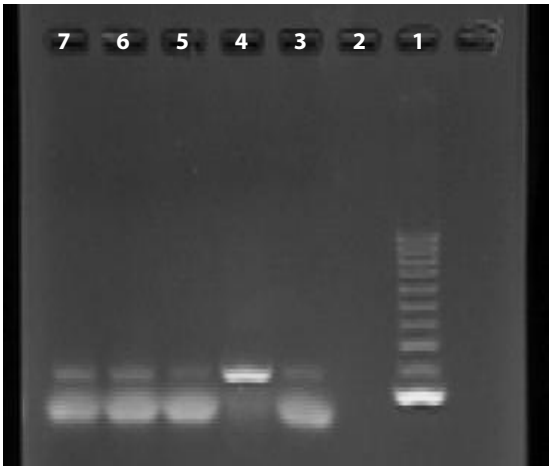

Figure 2. Agarose gel electrophoresis picture of Johne's nested PCR amplification. Lane 1: molecular weight standard (Bioline 100 bp Hyperladder), Lane 2: negative control lane 3 to lane 6 sample tested in duplicated (117 bp), Lane 7: positive control (*Mycobacterium avium* subsp. *paratuberculosis*).

indicate that Johne's disease is not a threat in the ruminant industry of Malaysia. As described in many reports, Johne's disease is a chronic and slow progressing disease, usually taking at least two years for infected animals to exhibit clinical signs. The slow development of clinical signs in MAP-infected animals might lead to misdiagnosis of the disease.

Moreover, the clinical signs produced by the disease were similar to clinical signs caused by other bacterial infections. Diarrhoea caused by Johne's disease is often misdiagnosed as other infection, such as salmonellosis, gastrointestinal parasitism and other chronic infectious diseases (Munir *et al*., 2014). Clinical signs such as poor body score and weakness are often found in other diseases and cannot be used as a definitive diagnosis.

Microbiological culture has been recognised as the most suitable test for diagnosis of Johne's disease in live animals. Its major advantage compared to PCR assay is that it can identify the infection 1 to 3 years prior to the appearance of clinical signs (Seyyedin *et al*., 2008). However, MAP being a fastidious and slow growing bacteria, its microbiological culture may take up to 16 weeks depending on laboratories (Martinson *et al*., 2008).

Decontamination of the samples is the biggest problem faced in the isolation of MAP. Being slow growing, it is outnumbered by other rapid growing bacteria or fungi including atypical mycobacterial species present in the faeces or intestinal specimens. Hence, efficient inactivation of undesirable contaminants is a crucial step for the successful isolation of MAP. The OIE (2014)

describes two methods. The first method uses oxalic acid and natrium hydroxide for decontamination, and isolation using Lowenstein-Jensen media. The second method used hexadecylpyridinium chloride for decontamination, and isolation using combination of HEY and Middlebrook 7H10.

VRI was using a decontamination method using acriflavine solution (National TB Centre, 1988). However, as a continuous effort in improving laboratory diagnostic techniques, VRI improved and adopted the technique recommended by OIE with slight modifications, i.e. using oxalic acid and natrium hydroxide solution for decontamination, followed by culturing on HEY containing mycobactin.

It has been reported that microbiological cultures sensitivity is low at the early stage of infection but may approach 100% in clinically infected animals (Gwozdz, 2010).

Another study found that culture from faecal samples are less reliable than tissue culture of small intestines and mesenteric lymph nodes due to the intermittent faecal shedding of MAP and contamination with other faster growing bacteria (Acharya *et al*., 2017). MAP is shed in the faeces of infected animals in all stages of the disease but at different levels (Stevenson *et al*., 2010). This low number of organisms in the faecal samples might lead to negative isolation particularly in young infected animals that have not started to shed the bacterium (Tiwari *et al*., 2006). The number of organisms excreted by animals is also dependent upon the stage of the disease in the individual animal (Well *et al*., 2006). Therefore, it is highly recommended to collect at least three samples at different times for isolation to confirm that the animals are free from Johne's disease.

PCR is not the most suitable to be used as definitive test to diagnose the disease, as it highly depends on the stage of infection at the time of diagnosis, quantity of DNA present in the samples, efficacy of protocols for DNA extraction, and the presence of inhibitors in the samples (Zapata *et al*., 2010). Although PCR assays are more specific and faster than conventional culture, it is less sensitive for detection of small numbers of organisms, perhaps due to inhibitory substances in faeces (Eamens *et al*., 2015). In most of the laboratories, including VRI, the role of PCR in diagnosis of Johne's disease has primarily been restricted to identification of culture isolates rather than direct detection from the samples (Eamens *et al*., 2015)

Most of the serological tests is recommended so as to obtain the prevalence of infection at the farm level. ELISA has been recommended by OIE for surveillance of Johne's disease in cattle. It is more sensitive and specific as compared to CFT. For sheep and goat, AGID is recommended.

The results of serology should be interpreted with care because even though the animals were positive, it does not indicate that the animals harbour the infection at that particular time. Detection of antibody titre might be also due to post vaccination exposure as reported in other countries. Moreover, serological tests cannot detect early stage infection because the host primary immune response is cell-mediated, with little or no involvement of humoral immunity (Stabel and Bannantine,

2005). Serologically positive animals should be confirmed by combination with other tests, such as isolation, microscopy and histopathology.

CONCLUSION

Microbiological culture of MAP remains as the gold standard for diagnosis of Johne's disease. Successful isolation of MAP is highly dependable on various factors including the methods of sampling, stages of infection and decontamination and isolation procedures. This study presents an improved technique for diagnosis of MAP through modification of OIE diagnostic tests.

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