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RESEARCH ARTICLE

Comparison of a Nested PCR-Enzyme-Linked Immunosorbent Assay with Various Diagnostic Tests for the Detection of *Mycobacterium avium* subsp. *paratuberculosis*

Jacky Peng-Wen Chan, Yi-Ju Chen and Chi-Young Wang*

Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 402, Taiwan
*Corresponding author: cyoungwang@dragon.nchu.edu.tw

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A B S T R A C T

The number of cases of Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is growing around the world. Detection of shed bacteria from faecal samples has been widely accepted as being an effective approach for the identification of infected cows, which, in turn, facilitates the implementation of quarantine procedures. A solid-phase nested PCR-enzyme-linked immunosorbent assay (PCR-ELISA) is reported here for detecting MAP. The primers and probes were designed to target the conserved region of insertion sequence 900 (*IS900*). The detection limit of this assay was ten copies of the *IS900* gene in a standard plasmid, which is equivalent to one copy of bacterial genomic DNA. A good linearity was observed in the standard curve across 10^5 orders of magnitude. No positive results were obtained when the test was carried out on *E. coli*, *Staphylococcus* sp., and *Enterococcus* sp., which are common pathogens isolated from cows in Taiwan. Thirty-one samples were tested by bacterial isolation, PCR, nested PCR, LAMP, nested PCR-ELISA, and serum ELISA in parallel. The results from the nested PCR-ELISA were consistent with those of a combination of bacterial culture and serum ELISA. In conclusion, this nested PCR-ELISA provides an option for laboratories with a thermocycler and an ELISA reader and can be used for both research and clinical purposes.

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INTRODUCTION

Currently, Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) still poses a severe problem throughout the world, resulting in enormous economic losses every year to the beef and dairy industries (Collins *et al*., 2010). Young calves often contract this disease via a faecal-oral route through the ingestion of colostrum or through contact with contaminated teats, milk, faeces, and the environment (Fecteau *et al*., 2013; Siddiqui *et al*., 2013). Those calves remain subclinically infected until the age of two to five years, and only low numbers of MAP are intermittently shed in milk and faeces (Eisenberg *et al*., 2013). Subsequently, severe weight loss, non-treatable diarrhoea, emaciation, decreased milk production, infertility, and eventual death occur. No clinical signs are noticed in these subclinically infected animals and therefore this group of animals becomes a persistent source that continue to introduce MAP into the environment. Therefore, an early effective diagnosis for MAP during the subclinical infection stage has become an important issue in the control of this disease. The presence of anti-MAP antibodies is often observed in clinical infected animals but may not be high enough to be detected in subclinical cases (Whitlock *et al*., 2000). Culture of MAP remains the "gold standard'' for diagnosis of Johne's disease (Sanftleben, 1990; Stabel, 1997), but decontamination of faeces often reduces the number of viable cells and, consequently, only approximately 50% of cattle infected with MAP can be accurately detected by culture.

DNA-based techniques that have been developed that target insertion sequence 900 (*IS900*); these include PCR, nested PCR, loop-mediated isothermal amplification (LAMP), and real-time PCR, and have provided practical means of detecting MAP (Enosawa *et al*., 2003; Khare *et al*., 2004; Leite *et al*., 2013). Confirmation of the

authenticity of the PCR products remains necessary and nucleotide hybridization with a specific probe is a convenient method compared to using sequencing analysis (Di Pinto *et al*., 2012). Therefore, a PCR-enzyme-linked immunosorbent assay (PCR-ELISA) based on the binding of a biotin-labelled probe is reported in this study. By means of both solution and solid-phase amplification, together with a colorimetric reaction involving streptavidin and peroxidase, such a system is able to detect MAP effectively.

MATERIALS AND METHODS

Isolation of MAP and serum ELISA: Faecal and serum samples were provided by the National Animal Health Research Institute of Taiwan or obtained from cattle on dairy farms in Taiwan. For MAP isolation, faecal sample was mixed with hexadecylpyridinium chloride (Sigma-Aldrich) and cultivated with Herrold's egg yolk agar slants for 16 weeks. Blood samples were tested by a MAP antibody test kit (IDEXX laboratories Inc., USA).

DNA extraction and construction of the standard plasmid: DNA was extracted from faecal sample using a QIAamp DNA stool mini kit (Qiagen). The bacteria were incubated at 37°C for 1 h in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.6% SDS, and 120 µg/ml proteinase K). Digestions were extracted with phenol-chloroform and then chloroform. The DNA was precipitated with ethanol. A standard plasmid, pT-Easy-ParaTB, containing the IS900 fragment was created. The copy number was calculated.

PCR, Nested PCR, LAMP, and Nested PCR-ELISA: The primers and the probe are presented in Table 1. The PCR mixture contained 2 µl of template DNA, 2.5 µl of 10 x buffer, 0.2 mM of each dNTP, 0.4 µM F3 and B3 primers, and 0.5 µl of Taq polymerase. The reactions included 94°C for 10 min, 36 cycles composed of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and 72°C for 10 min. The reaction for the nested PCR mixture including 1 µl of PCR product as the template and the InF and InR primers was prepared as above using the same program with 57°C as an annealing temperature. The LAMP mixture contained 0.8 µM FIP and BIP primers, 0.4 µM F3 and B3 primers, 0.8 mM each dNTP, 20 mM Tris-HCl, 1 x ThermoPol buffer, 8 U of *Bst* DNA polymerase (New England Biolabs), and template DNA. The amplification was performed at 65°C for 60 min. PCR, nested PCR, and LAMP products were analysed by agarose gels. For nested PCR-ELISA, 100 nM InF-T primer in 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 10 mM imidazole was added to each well (Nalge Nunc International, USA) for an incubation at 50°C. The wells were washed with DIAPOPS (Detection of Immobilised Amplified Product in a One-Phase System) buffer (80 mM Tris-HCl, 20 mM Tris base, 150 mM NaCl, and 0.1% Tween 20). After blocking, the nested PCR reactions consisted of 1 µl of PCR products, 2.5 µl of 10 x buffer, 0.4 mM of each dNTP, 0.4 µM InF primer, 3.2 µM InR primer, and 0.5 µl of Taq polymerase and amplification was carried out following the above program. Non-covalently bound amplicons were denatured by washes in 0.2 M NaOH with 0.1% Tween 20. Probe in hybridization buffer (5 x standard saline citrate, 5 x Denhardt's solution, and 0.1% Tween 20) was used for the hybridisation at 50°C for 1 h. Streptavidin-peroxidase was added to each well for an incubation at 37°C for 1 h. Finally, ABTS was added and the OD_{405} nm was measured. The mean OD_{405} of the negative samples plus three standard deviations was defined as the cut-off value.

Sensitivity and specificity of the PCR, Nested PCR, LAMP and Nested PCR-ELISA and comparison of detection rates of samples using various diagnostic methods: The sensitivity of the various methods was examined using different amounts of the pT-Easy-ParaTB plasmid as template. A standard curve of the nested PCR-ELISA system was created. The specificity of the various methods was examined using *E. coli*, *Staphylococcus* sp., and *Enterococcus* sp.. All samples were tested using various methods in parallel. The positive detection rate was calculated and compared with each other by Chi-square analysis. Significant differences were defined as $P<0.05$.

RESULTS

Sensitivity of the PCR, Nested PCR, LAMP, and Nested PCR-ELISA methods: A 169-bp product was obtained from 10 ng, 1 ng, and 0.1 ng of the pT-Easy-ParaTB standard plasmid per reaction using PCR. This is equivalent to 10^4 copies of the *IS900* gene or 10^3 copies of the genomic DNA of MAP per reaction (Fig. 1A). Using nested PCR, a 120-bp product was detected from 10 ng, 1 ng, 0.1 ng, 10 pg and 1 pg of the standard plasmid per reaction. This is close to 10^2 copies of the *IS900* gene or 10 copies of genomic DNA of MAP per reaction (Fig. 1B). Similarly, as little as 1 pg of the standard plasmid was able to be detected by LAMP per reaction, which is equivalent to 10^2 copies of the *IS900* gene or 10 copies of MAP genomic DNA per reaction (Fig. 1C). After 15 min of the substrate reaction, the intensity of the green colour of positive samples became readily identifiable by eye when using the nested PCR-ELISA. Positive results were obtained from 10 ng, 1 ng, 0.1 ng, 10 pg, 1 pg, and 0.1 pg per reaction. No amplification was detected with the negative controls and the detection limit of the PCR-ELISA was equivalent to 10 copies of the *IS900* gene or 1 copy of MAP genomic DNA per reaction (Fig. 1D). In addition, a standard curve was generated between the value of OD_{405} nm and $\log_{10}$ amount of the standard plasmid. The equation of the regression line was $y=0.2236x+0.2452$, with an R-square of 0.96. The average mean OD_{405} values for 0.1 pg, 1 pg, 10, pg, 0.1 ng, 1 ng, and 10 ng per reaction were 0.309±0.049, 0.852±0.093, 0.950±0.107, 1.122±0.174, 1.434±0.237, and 1.492± 0.255, respectively. Therefore, the cut-off value of 0.26 was close to 0.258, which had been empirically defined from the average mean of the OD_{405} nm of all negative samples plus three standard deviations.

Specificity of the PCR, Nested PCR, LAMP and Nested PCR-ELISA methods: PCR, nested PCR and LAMP were positive only for the genomic DNA of MAP. No amplification of genomic DNA from *E. coli*, *Staphylo-*

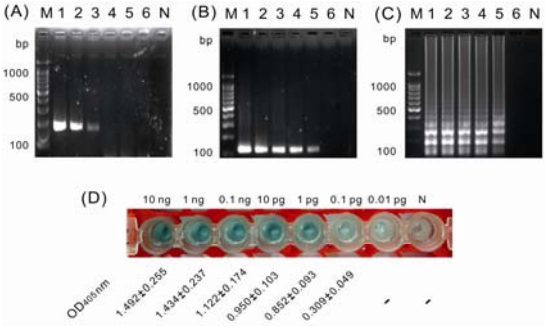

Fig. 1: The sensitivity of detection of *Mycobacterium avium* subsp. *paratuberculosis* using PCR (A), nested PCR (B), LAMP (C), and nested PCR-ELISA (D). The pT-Easy-ParaTB standard plasmid was used as the template. M: DNA marker, lanes 1-6 (1 ng, 0.1 ng, 10 pg, 1 pg, 0.1 pg, and 0.01 pg), and N: negative control.

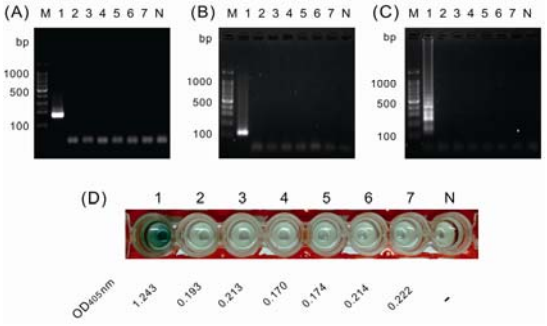

Fig. 2: The specificity of detection of *Mycobacterium avium* subsp. *paratuberculosis* using PCR (A), nested PCR (B), LAMP (C), and nested PCR-ELISA (D). M: DNA marker, lane 1: MAP, lane 2: *E. coli* (duplicated No. 1), lane 3: *E. coli* (duplicated No. 2), lane 4: *Staphylococcus* sp. (duplicated No. 1), lane 5: *Staphylococcus* sp. (duplicated No. 2), lane 6: *Enterococcus* sp. (duplicated No. 1), lane 7: *Enterococcus* sp. (duplicated No. 2), and N: negative control.

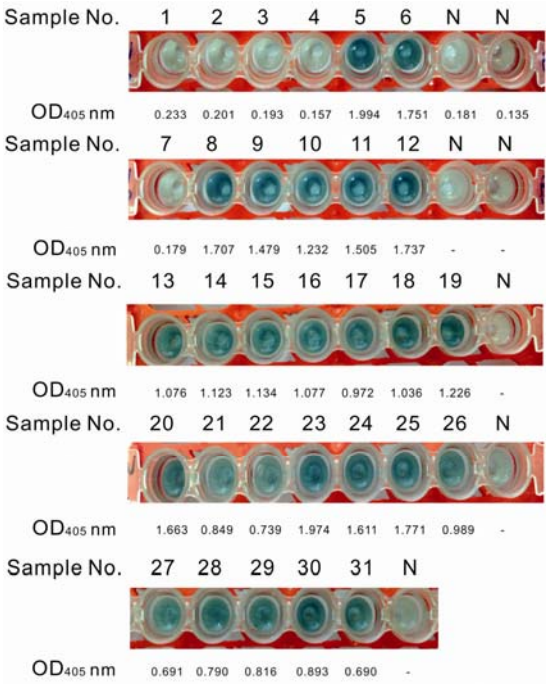

Fig. 3: The results obtained using the cattle samples using the nested PCR-ELISA test. The OD_{405} nm value of each sample is represented below the colorimetric result. N indicates the negative controls.

coccus sp., and *Enterococcus* sp. was observed using duplicate samples (Fig. 2A, 2B, and 2C). Using nested PCR-ELISA, the OD_{405} values for MAP, *E. coli*-1, *E. coli*-2, *Staphylococcus* sp.-1, *Staphylococcus* sp.-2, *Enterococcus* sp.-1, *Enterococcus* sp.-2, and the negative control were 1.243, 0.193, 0.213, 0.170, 0.174, 0.214, 0.222, and 0.197, respectively (Fig. 2D). Therefore, no positive results were detected using the non-MAP bacteria.

Comparison of the detection rates of samples using various diagnostic methods: Of the thirty-one samples, 18, 24, 29, 26, and 26 samples were positive by bacterial culture, PCR, nested PCR, LAMP, and nested PCR-ELISA, respectively. Samples No. 1, No. 2, No. 4, and No. 7 were always negative for bacterial culture, PCR, LAMP, and nested PCR-ELISA but were positive for nested PCR. Sample No. 3 was negative for all assays. Samples No. 5, No. 6, No. 8-16, and No. 19-21 were positive using bacterial culture, PCR, LAMP, nested PCR, and nested PCR-ELISA. Samples No. 17 was positive using bacterial culture, LAMP, and nested PCR-ELISA but not using PCR and nested PCR. Sample No. 18 was positive using bacterial culture, nested PCR, LAMP, and nested PCR-ELISA but not using PCR. MAP can be successfully cultivated from samples No. 17 and No. 18. Samples No. 22 and No. 25 were positive using bacterial culture, ELISA, PCR, LAMP, nested PCR, and nested PCR-ELISA. Although no positive results were obtained using bacterial cultures with samples No. 23, No. 24, and No. 26-31, all nucleic acid-based results and ELISA were positive for these samples (Table 2). After Chi-square analysis, a significant difference was only found between bacterial culture and the other molecular diagnostic methods. The average copy number of MAP in the samples negative by culture but positive by molecular biology was $10^{2.9}$.

DISCUSSION

A highly sensitive *Mycobacterium tuberculosis* assay using PCR-ELISA based on the addition of labelled-PCR products that hybridised with biotinylated probe and that used plates pre-coated with streptavidin has been described (Cho *et al*., 1995; Yam *et al*., 2004). This report differs from this method which describes a solid-phase PCR-ELISA based on DIAPOPS technology. Compared to the earlier method, this strategy has several advantages, which include simultaneous interfacial and surface amplifications during the PCR, a good linearity between the amount of product and the DNA template, and a closed well format that allows both amplification and detection using a single setup (Adessi *et al*., 2000; Raji *et al*., 2011). In addition, linearity across at least 10^5 orders of magnitude of the template was found in this study. The specificity of this assay was verified using *E. coli*, *Staphylococcus* sp., and *Enterococcus* sp.. On the basis of an epidemiological analysis over a number of years, these bacteria, in addition to MAP, are the most common pathogens found on dairy farms in Taiwan; furthermore, other *Mycobacterium* sp. has only been reported in other animals.

Nested PCR results of samples No. 1, No. 2, No. 4, and No. 7 were likely to be false positive. This was

Table 1: Primers used for the detection of *Mycobacterium avium* subsp. *paratuberculosis*

Primer name	Type	Sequence (5'-3')	Length (bp)	Nucleotide position
InF[bdg]	Forward inner	GGATGGCCGAAGGAGATTGG	20	93-112
InR[bdg]	Reverse inner	TCCAGATCAACCCAGCAGAC	20	193-212
F3[abcdgh]	Forward outer	GGGTATGGCTTTCATGTGGT	20	64-83
B3[abcdgh]	Reverse outer	CACCTCCGTAACCGTCATT	19	214-232
FIP (F1+F2c)[cgh]	Forward inner	TCAATTAGCGGTCGAGTCGTCGTTGGATGGCCGAA GGAGAT	41	F1:129-149 F2c:586-604
BIP(B1+B2c)[cgh]	Reverse inner	TGCGATTGGATCGCTGTGTATCCAGATCAACCCAG CAGAC	40	B1:684-702 B2c:738-758
InF-T[deg]	Forward Solid-Phase	TTTTTTTTTGGATGGCCGAAGGAGATTGG	30	$dT_{(10)}$ and 93-112
Probe[dfg]	Reverse	ACGCCGACGTGTCCTTACACAGCGAT	26	163-188

[a] PCR primers; [b] nested PCR primers; [c] LAMP primers; [d] nested PCR-ELISA primers; [e] primer modified with 5'-terminal phosphorylation; [f] Probe modified with 5'-terminal biotin; [g]Nucleotide position based on the Genbank accession number X16293; [h] Primers adapted from Enosawa *et al.* (2003)

Table 2: Detection of *Mycobacterium avium* subsp. *paratuberculosis* using bacterial culture, ELISA, PCR, nested PCR, LAMP, and nested PCR-ELISA

Sample No.	Bacterial culture	ELISA	PCR	Nested PCR	LAMP	Nested PCR-ELISA			
						Results	$OD_{405\ (nm)}$	DNA conc. (pg)	The estimated copy number of bacteria
1	-	N.D.	-	+/-[a]	-	-	0.233	$<10^{-1}$	-
2	-	N.D.	-	+/- [a]	-	-	0.201	$<10^{-1}$	-
3	-	N.D.	-	-	-	-	0.193	$<10^{-1}$	-
4	-	N.D.	-	+/- [a]	-	-	0.157	$<10^{-1}$	-
5	+	N.D.	+	+	+	+	1.994	$10^{5.5}$	$10^{7.5}$
6	+	N.D.	+	+	+	+	1.751	$10^{4.5}$	$10^{6.5}$
7	-	N.D.	-	+	-	-	0.179	$<10^{-1}$	-
8	+	N.D.	+	+	+	+	1.707	$10^{3.4}$	$10^{6.3}$
9	+	N.D.	+	+	+	+	1.479	$10^{2.3}$	$10^{5.4}$
10	+	N.D.	+	+	+	+	1.232	$10^{3.5}$	$10^{4.3}$
11	+	N.D.	+	+	+	+	1.550	$10^{4.4}$	$10^{5.5}$
12	+	N.D.	+	+	+	+	1.737	$10^{1.7}$	$10^{6.4}$
13	+	N.D.	+	+	+	+	1.077	$10^{1.3}$	$10^{3.7}$
14	+	N.D.	+	+	+	+	0.972	$10^{1.5}$	$10^{3.3}$
15	+	N.D.	+	+	+	+	1.036	$10^{2.3}$	$10^{3.5}$
16	+	N.D.	+	+	+	+	1.226	$10^{4.1}$	$10^{4.3}$
17	+	N.D.	-	-	+	+	1.663	$10^{0.8}$	$10^{6.1}$
18	+	N.D.	-	+	+	+	0.849	$10^{0.3}$	$10^{2.8}$
19	+	N.D.	+	+	+	+	0.739	$10^{5.4}$	$10^{2.3}$
20	+	N.D.	+	+	+	+	1.974	$10^{3.9}$	$10^{7.4}$
21	+	N.D.	+	+	+	+	1.611	$10^{4.6}$	$10^{5.9}$
22	+	+	+	+	+	+	1.771	$10^{1.3}$	$10^{6.6}$
23	-	+	+	+	+	+	0.989	$10^{1.7}$	$10^{3.3}$
24	-	+	+	+	+	+	1.076	$10^{1.9}$	$10^{3.7}$
25	+	+	+	+	+	+	1.123	$10^{1.9}$	$10^{3.9}$
26	-	+	+	+	+	+	1.134	$10^{1.9}$	$10^{3.9}$
27	-	+	+	+	+	+	0.691	$10^{0.1}$	$10^{2.1}$
28	-	+	+	+	+	+	0.790	$10^{0.5}$	$10^{2.5}$
29	-	+	+	+	+	+	0.816	$10^{0.6}$	$10^{2.6}$
30	-	+	+	+	+	+	0.893	$10^{1.0}$	$10^{3.0}$
31	-	+	+	+	+	+	0.690	$10^{0.1}$	$10^{2.1}$
The P value of the χ^2 analysis	0.369[b]	N.D.	0.002[b]	0.000[b]	0.000[b]			0.000[b]	

[a]This sample was a false-positive; [b]Significant difference for other methods *vs.* bacterial culture

supported by both sequence analysis and negative results of the nested PCR-ELISA, which involves hybridisation with a specific probe. It may be caused by an accidental contamination. The PCR-negative samples (No. 17 and No. 18) still were able to be cultivated successfully for MAP because the detection limit for PCR is less sensitive and the sample volume of the HEYA culture can be up to 200 times larger than the actual volume used for PCR after correction. As a result of the amplification effect of a positive signal via the colorimetric reaction, the nested PCR-ELISA is more sensitive than the nested PCR, which explains the results obtained with sample No. 18. Therefore, a thorough dispersion of the samples before extraction of the DNA seems to be necessary. The findings with samples No. 23, No. 24, and No. 26-31 are consistent with the results of a study in the United Kingdom and can be explained if the collection time of the faecal samples was not optimal (Millar *et al*., 1996). This is because the shedding of bacteria into faeces is intermittent and can also vary in amount; only viable complete bacteria can be successfully isolated. The viability of MAP bacteria is reduced during the decontamination step that is used at the National Animal Health Research Institute in Taiwan (Stabel, 1997). However, the presence of antibody against MAP in these cattle pinpoints their infectious status (Collins *et al*., 1994). The ELISA serum test is routinely used in the screening program for MAP across Taiwanese dairy farms and is able to provide additional evidence as to the infectious status of a given animal. The average copy number of MAP in the culture-negative samples but positive by other DNA-based methods remains a low level compared to the other tested samples. This may indicate why MAP was unable to be isolated from these low level MAP samples.

There are numerous advantages to the use of PCR-ELISA, including the detection of products without the use of agarose gel electrophoresis, the ability to directly observe the intensity of the colorimetric reaction using the naked eye, the possibility of quantifying the amplified

products by the spectrophotometer after colour development, and the ability to use a large-scale screening platform using 96 well sample plates (Wilson *et al*. 2002). Compared to an immunomagnetic PCR-ELISA for MAP, which is based on a separation of bacteria from the other bacteria and material using monoclonal anti-MAP antibody, this nested PCR-ELISA is simple and user-friendly (Metzger-Boddien *et al*., 2006). In addition, the detection limits of the immunomagnetic PCR-ELISA and the nested PCR-ELISA were 10-20 and 1 MAP genome, respectively, which revealed the superiority of the latter method (Metzger-Boddien *et al*., 2006).

Conclusion: Despite recent advances in molecular diagnostic techniques such as LAMP and real-time PCR, which offer sensitive, rapid, and robust methods for the detection of MAP, this nested PCR-ELISA remains an inexpensive choice for laboratories with only a thermocycler and an ELISA reader and can be used for both research and clinical purposes.

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